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May 22, 2006

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May 22, 2006	Ron J. Laby
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Mail Stop Appeal Brief-Patents
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Re: *SN 09/989,739 "MAIZE CYTOPLASMIC GLUTAMINE SYNTHETASE PROMOTER COMPOSITIONS AND METHODS FOR USE THEREOF" by Brendan Hinchey, et al.; Our Ref. DEKM:177US; Client Ref. 52212 US 00*

Commissioner:

Enclosed for filing in the above-referenced patent application is:

1. An Appeal Brief;

2. A check for $500.00 to cover the Appeal Brief filing fee; and

3. A return postcard to acknowledge receipt of these materials. Please date stamp and mail this postcard.

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Respectfully submitted,

Ron J. Laby, Reg No. 53,173, for

Robert E. Hanson
Reg. No. 42,628

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Brendan Hinchey and Hee-Sook Song

Serial No.: 09/989,739

Filed: November 20, 2001

For: MAIZE CYTOPLASMIC GLUTAMINE SYNTHETASE PROMOTER COMPOSITIONS AND METHODS FOR USE THEREOF

Group Art Unit: 1638

Examiner: Cathy K. Worley

Atty. Dkt. No.: DEKM:177US

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May 22, 2006 — Date

Ron J. Laby

BRIEF ON APPEAL

TABLE OF CONTENTS

PATENT

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief. The date for filing this Brief is May 22, 2006. The fees for filing this Appeal Brief are attached. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/DEKM:177US.

Please date stamp and return the attached postcard as evidence of receipt.

I. REAL PARTY IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

II. RELATED APPEALS AND INTERFERENCES

Related appeals are Appeal Nos. 2003-0936 (Serial No. 09/532,806, now U.S. Patent No. 6,747,189) and 2005-0409 (Serial No. 09/757,089, now allowed). Copies of the Board decisions issued in these cases are provided under the Related Proceedings Appendix. While these cases are not related by priority to the current case, they involved many of the same written description and enablement issues presented in the current appeal, share the same Real Party in Interest with the current case, and involved substantially similar specifications and claims relative to the current case. It is therefore believed that these appeals will have a bearing on the current appeal.

III. STATUS OF THE CLAIMS

Claims 1-89 were filed with the application. Claims 2-3 were canceled during prosecution. Claims 1 and 4-89 are therefore currently pending. Claims 1 and 4-89 were rejected by the Examiner in the Final Action and are the subject of this appeal. Claim 1 was amended in Appellants' Response to Final Office Action filed March 16, 2006, which was subsequently entered by the Examiner. A copy of the appealed claims as they currently stand is included in Section VIII.

IV. STATUS OF AMENDMENTS

Claim 1 was amended in the Response to Final Office Action filed March 16, 2006. The Amendment was entered by the Examiner on April 3, 2006. The copy of the appealed claims found in Section VIII reflects the Amendment.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention relates to promoter sequences isolated from the maize cytoplasmic glutamine synthetase (GS_{1-2}) gene and fragments thereof having promoter activity. Specification at page 7, lines 1-10. The claimed invention further relates to transgenic plants and seeds comprising such a promoter sequence. Specification at page 7, lines 17-24. The claimed invention further relates to methods for preparing transgenic plants and plant breeding involving use of the promoter sequences. Specification at page 11, lines 16-30.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

(A) Are claims 1 and 4-89 properly rejected as failing to comply with the written description requirement under 35 U.S.C. §112, first paragraph?

(B) Are claims 1 and 4-89 properly rejected as lacking enablement under 35 U.S.C. §112, first paragraph?

(C) Are claims 1, 11-13, 15, 17-18, 20, 22, 24-25, 28-30, 34-37, 57-66, 69-70, and 72 properly rejected under 35 U.S.C. § 102(e) as being anticipated by Muhitch (U.S. Patent Publ. No. US20040148651)?

(D) Are claims 1 and 11-89 properly rejected under 35 U.S.C. § 103(a) as obvious over Muhitch in combination with prior art references?

VII. ARGUMENT

A. Substantially Identical Issues Were Decided by the Board in Appeal No. 2003-0936

Appellants note that, prior to the appeal of the current case, substantially identical issues were decided by the Board in the favor of the current Real Part in Interest in Appeal No. 2003-0936 (U.S. Ser. No. 09/532,806; "the '806 application"). This application was assigned to DEKALB Genetics Corporation, which is a wholly owned subsidiary of the Real Party in

Interest, Monsanto Company. A copy of the previous Decision is attached in the Related Proceedings Appendix below. A subsequent Board decision in another promoter case that also presented these same issues (Appeal No. 2005-0409; U.S. Ser. No. 09/757,089) was subsequently remanded to the Examiner with an order to distinguish the case on the facts or the law from Appeal No. 2003-0936, which the Examiner did not do. This case has now been allowed.

Appeal No. 2003-0936 concerned written description and enablement rejections made to claims directed to a maize promoter sequence and was handled by the same art unit as the current case. The main independent claim on appeal read as follows:

> 1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

The main independent claim here reads as follows:

> 1. An isolated nucleic acid sequence comprising a cytoplasmic glutamine synthetase GS_{1-2} promoter, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises:
>
> (a) a nucleic acid sequence of SEQ ID NO:18 or a fragment thereof, having promoter activity, wherein the fragment comprises from 400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18; or
>
> (b) a nucleic acid sequence comprising from 400 to 2547 contiguous nucleotides that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes.

As can be seen, although the wording of the claims is somewhat different, they are substantially similar to the current claims absent the particular promoter claimed. Both claims recite a maize promoter that comprises a minimum length of contiguous nucleotides (bases) of the full length promoter sequence, which was 3536 bp in the case of the '806 application and is 2547 bp in the case of the current application. While the current claims also encompass nucleic acids comprising from 400 to 2547 contiguous nucleotides that hybridize to the nucleic acid

sequence of SEQ ID NO:18 under specific stringency conditions, these conditions are highly stringent permitting minimal variability and further the 400 bp recited in the claim is substantially longer than the 95 bp minimal fragment recited in the '806 application claim. The specifications and teachings of the two applications are also substantively identical, as the '806 application served as a template for the drafting of the current application and generally includes the same disclosure.

Essentially the same reasoning was applied by the Examiner in Appeal No. 2003-0936 as in this appeal. For example, the Examiner asserted that the claims lacked written description because certain functional elements within the full length promoter sequence were not specified, *e.g.*, that Appellants did not describe how or why the claimed invention works. An enablement rejection was also issued on the basis that, because of the lack of recitation of the functional elements, only the full length sequence was enabled.

The Board reversed the Examiner on both rejections. The Board first noted that the claims were supported by a literal written description in the sequence listing. The Board specifically noted the following:

> we find that here the isolated nucleic acid maize GRP promoter appellants claim comprising at least 95, 110,125, 250, 400, 750, 1000, 1500, 2000, 2500, 3000, and 3536 contiguous nucleotide fragments of the 3536 contiguous nucleotides of SEQ ID NO:1 of Figure 4 is so precisely defined in terms of structure, formula, chemical name, and function, including Figures 1-4 and examples, that persons skilled in the art immediately would have understood what appellants claim as their invention and could readily distinguish what appellants claim from anything that is in common use, all that is known, and anything proposed for production and use in the art.
>
> Decision at 2, p. 12.

The Board therefore held that the test for written description applied by the Examiner was not the correct one, and found that the written description requirement was satisfied by the literal description in the specification.

The Board then analyzed the enablement rejection by first noting that the field was unpredictable and experimentation would be required to make and use promoter sequences shorter than full length, but finding that substantial guidance was provided in the specification and the advanced knowledge in the art rendered any experimentation routine and not undue. Decision at p. 22-24. The teaching pointed to by the Board as supporting enablement is also found in the current application. Specifically, the current application includes the following: detailed teaching regarding the preparation of derivatives of the full length promoter sequence (page 15, line 21 to page 19, line 30); plant transformation constructs comprising the promoter compositions and elements for inclusion in these constructs (page 20, line 1 to page 66, line 12), assays of gene expression (p. 66, line 15 to page 74, line 12); numerous plant transformation methods (page 75, line 1 to page 81, line 22); methods for culturing recipient cells for transformation (page 81, line 25 to page 89, line 21); and production and characterization of stably transformed plants, including methods for assaying for transgene expression (page 89, line 25 to page 99, line 11).

The working examples in particular show the following: isolation of the cytoplasmic glutamine synthetase GS_{1-2} promoter (Example 1); construction of transformation constructs comprising the cytoplasmic glutamine synthetase GS1-2 promoter (Example 2); transformation of maize tissues (Examples 3-4); regeneration of fertile transgenic plants (Example 5); and analysis of GUS reporter gene activity to confirm the extent of promoter GS1-2 promoter expression (Example 6, Table 8). Further detailed teaching of the methods for producing and screening promoter constructs is described in the Detailed Description of the Invention. For example, this section describes (1) selection of transformants, (2) regeneration of transgenic plants and seed production, and (3) genetic characterization to confirm transgenic plants,

including confirmation of the presence and expression of transgenic sequences. Screening techniques for quantifying expression are described that allow the quantitation and detection of RNA produced from introduced genes, as well as by the expression of screenable marker genes as is described in the working examples.

The foregoing guidance is as complete as the substantial teaching found enabling in the '806 application, despite the fact that the '806 application promoter was substantially longer than the currently claimed promoter. The Board's decision in the '806 application was therefore made on the same legal issues under the same factual background. While the previous decision is not binding precedent, there is no basis for this Appeal to be viewed in a manner different than the '806 application. Appellants therefore respectfully request that the same reasoning be applied in this case and the written description and enablement rejections be reversed accordingly.

B. <u>The Claims Comply with the Written Description Requirement Under 35 U.S.C. 112, First Paragraph</u>

The Examiner rejects claims 1 and 4-89 for failing to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. Specifically, the Examiner alleges that the claims contain subject matter which was not described in the specification in such a way as to convey possession of the invention at the time the application was filed. The rejection should be reversed as explained below.

1. Written Description Must Be Analyzed with Respect to the Claimed Invention

The rejection appears to require Applicants to show how or why the claimed promoter sequences function. In particular, the Examiner suggests that Applicants must show which

structural features are necessary for the function of the glutamine synthetase $GS_{1\text{-}2}$ promoter. However, what is relevant under 35 U.S.C. § 112, first paragraph is that Applicants were in possession of the ***claimed invention***; and not unclaimed features envisioned in the Action. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991).

The claims are not directed to particular functional elements. For example, claim 1 of the application currently reads as follows:

1. An isolated nucleic acid sequence comprising a cytoplasmic glutamine synthetase $GS_{1\text{-}2}$ promoter, wherein the cytoplasmic glutamine synthetase $GS_{1\text{-}2}$ promoter comprises:

   (a) a nucleic acid sequence of SEQ ID NO:18 or a fragment thereof, having promoter activity, wherein the fragment comprises from 400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18; or

   (b) a nucleic acid sequence comprising from 400 to 2547 contiguous nucleotides_that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes.

Thus what is relevant for purposes of written description is that Applicants teach at least 400 nucleotides of SEQ ID NO:18. Applicants have explicitly done so by providing the nucleic acid sequence of SEQ ID NO:18 in the sequence listing and in FIG. 2, both of which were provided with the specification as filed. Applicants do not lack a written description for what is expressly set forth in the application. While the claims encompass nucleic acid sequences that hybridizes to SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes, these conditions are stringent and thus define a subset of sequences fully described by SEQ ID NO:18. It is well settled that the Applicants need not provide an *ipsis verbis* description for the claimed invention. *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) (stating that the written description requirement does not require an applicant to "describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed" (citations omitted)). Here, the

entire scope of claimed subject matter is supported by the literal description in the sequence listing. *The Regents of The University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1568; 43 USPQ2d 1398, 1406 (Fed. Cir. 1997) (noting that a name alone does not satisfy the written description requirement where "it does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, *as one can do with a fully described genus, visualize or recognize the identity of the members of the genus*" (emphasis added)). All of the claimed subject matter has therefore been fully described pursuant to 35 U.S.C. § 112, first paragraph.

2. The Holding of *Fiers* is Inapplicable to the Current Claims

In the instant case, the independent claims recite a minimum of 400 contiguous nucleotides of SEQ ID NO:18. Nonetheless, the Examiner relies on *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993) for the proposition that the instant claims lack written description. However, *Fiers* is not applicable to the instant situation and involves a fact pattern distinct from the current situation. *Fiers* involved a three-way interference proceeding over DNA coding for a human fibroblast interferon polypeptide. With respect to the written description requirement, the three parties were entitled to the priority date of their foreign applications only if those applications disclosed the nucleotide sequence of the claimed interferon gene. One of the parties' foreign applications could not be used to establish priority because it merely disclosed a method that might be used to obtain mRNA coding for the claimed interferon gene.

Here, however, the Applicant's specification has provided the claimed nucleic acid sequences by way of SEQ ID NO:18. Applicants have further provided a written description of the subfragments of SEQ ID NO:18. For example, at page 7, lines 4-10, subfragments of SEQ

ID NO:18 are described comprising at least about 135, 250, 400, 750, 1000, 1500, 1750, 2000, 2250, and 2500 contiguous nucleotides up to the full 2547 nucleotides of SEQ ID NO:18. These sequences all have literal support in the sequence listing. In view of the nucleic acid sequence of SEQ ID NO:18 provided in the sequence listing and in FIG. 2, there is no basis to allege that the Applicants did not clearly convey possession of the claimed invention.

In view of the foregoing, Applicants assert that the specification satisfies the written description requirement. Reversal of the rejection under 35 U.S.C. § 112, first paragraph is thus respectfully requested.

C. <u>The Claims Are Enabled Under 35 U.S.C. 112, First Paragraph</u>

The Examiner rejects claims 1 and 4-89 as not being enabled by the specification. In particular, the Action alleges that the specification is enabling only for the entire 2547 nucleotide sequence of SEQ ID NO:18. The rejection should be reversed as explained below.

1. The Enablement Requirement Must Be Applied With Respect to the Claimed Invention

The Examiner alleges that the specification does not disclose certain structural and functional information regarding which fragment derived from or hybridizing to SEQ ID NO:18 would be likely to have promoter function. However, Applicants note that which structural or functional elements are present is irrelevant. Applicants need not describe why or how the invention works. All that is required under 35 U.S.C. §112, first paragraph, is that the specification teaches one reasonably skilled in the art how to make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That is, Applicants must only enable what is claimed. See *Durel Corp. v. Osram Sylvania Inc.*, 256 F.3d 1298, 1306-07 (Fed. Cir. 2001). Here, the claimed invention represents contiguous sequences of 400 nucleotides or more of SEQ ID NO:18, the use thereof and

compositions produced therefrom. As described below, the specification has fully enabled this subject matter.

2. Applicants Have Affirmatively Demonstrated Compliance With the Enablement Requirement

The current claims recite nucleic acids comprising at least 400 contiguous nucleotides of SEQ ID NO:18. Provided in the Sequence Listing of the specification is the nucleic acid sequence of SEQ ID NO:18. This is more than adequate to fully enable one of skill in the art to prepare nucleic acid sequences of at least 400 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. The Examiner, however, suggests that one of skill in the art would be without guidance in obtaining the claimed fragments of this sequence because the specification does not provide the "minimal functional promoter regions derived from SEQ ID NO:18." However, the structural information of this subject matter is the sequence itself, which is given in SEQ ID NO:18.

The Examiner further ignores extensive teaching in the specification that goes well beyond what is required under the first paragraph of 35 U.S.C. § 112. For example, provided in the specification from page 16, line 18, to page 19, line 30, is detailed teaching regarding the preparation of derivatives of the full length promoter sequence. Described from page 20, line 1, to page 31, line 14, of the specification are plant transformation constructs comprising the promoter compositions, as well as elements for inclusion in these constructs. Described in detail from page 75, line 1, to page 81, line 15 are numerous methods that are known to those of skill in the art for transforming plants including, for example, direct delivery of DNA by PEG-mediated transformation of protoplasts, desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by *Agrobacterium*-mediated transformation and acceleration of DNA coated particles. Described from line 23, page 81 to line 21, page 89 are

methods for culturing recipient cells for transformation. Described from page 89, line 25, to page 99, line 11, are methods for the production and characterization of stably transformed plants, including methods for assaying for transgene expression.

In the working examples, Example 1 at page 108 describes the isolation of the maize pedicel-specific glutamine synthetase GS_{1-2} promoter from genomic DNA of *Zea mays* variety 01IBH2 using an inverse PCR strategy with primers designed from the glutamine synthetase GS_{1-2}. A 2.7 Kb fragment containing the glutamine synthetase GS_{1-2} promoter region was identified.

Example 2 at page 112 describes the construction of transformation constructs comprising the glutamine synthetase GS_{1-2} promoter. In Example 4, the bombardment of H99 immature embryos with constructs comprising the glutamine synthetase GS_{1-2} promoter is described. In particular, the *uidA* (GUS) reporter gene was fused to the glutamine synthetase GS_{1-2} promoter for evaluation of expression stable transformation experiments. Example 5 describes the regeneration of transgenic plants from transformed H99 maize cells.

Still further, Example 6 at page 117 describes analysis of glutamine synthetase GS_{1-2} promoter expression in transgenic maize. Small regenerated plants from a number of independent transformation events were assayed for GUS activity by histochemical staining and were positive for *uidA* reporter gene expression. The R_0 plants were crossed to a proprietary inbred line (H99) or were self pollinated and resultant R_1 plant progeny, as well as progeny of subsequent generations, were analyzed for GUS expression. GUS expression, as directed by the glutamine synthetase GS_{1-2} promoter, was observed in the cob vasculature, pedicel, basal conducting cells, and silk scar (Table 8).

Further, other detailed teaching for the transformation and assaying of plants with selected nucleic acids is contained in the remaining examples. In particular, Example 3 at page 113 teaches the preparation of microprojectiles in significant detail, while Example 7 at page 119 teaches *Agrobacterium tumefaciens*-mediated transformation.

Further detailed teaching of the methods for producing and screening promoter constructs is described in section VII of the Detailed Description of the Invention, entitled "Production and Characterization of Stably Transformed Plants" at pages 89-99. For example, this section describes (1) selection of transformants, (2) regeneration of transgenic plants and seed production, and (3) genetic characterization to confirm transgenic plants, including confirmation of the presence and expression of transgenic sequences. Screening techniques for quantifying expression are described that allow the quantitation and detection of RNA produced from introduced genes, as well as by the expression of screenable marker genes as is described in the working examples. The foregoing teachings in Applicants' specification are more than adequate to enable the full scope of the invention and cannot properly be ignored. *In re Wands*, 858 F.2d at 737.

It would be a straightforward matter for one of skill in the art to identify subfragments of SEQ ID NO:18 having promoter activity, especially given the detailed teachings in the specification. While Applicants acknowledge this would require some routine screening, "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d at 737. Furthermore, some amount of experimentation is permissible, especially when the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.* (quoting *Ex parte Jackson*, 217 USPQ2d 804, 807 (Bd. App. 1982)). The detailed teaching in the specification has

provided all of the methodology necessary for creating and screening the subject subfragments for promoter activity.

Given the fact that SEQ ID NO:18 is only 2547 nucleic acids long, nothing could be further from the truth than to suggest that it would require undue experimentation to create subfragments of this sequence and screen them for activity. This is underscored by the fact that the working examples provide exactly the methodology that one could use to screen subfragments for activity, namely inserting promoter fragments of SEQ ID NO:18 into a transformation construct, as described in Example 2 for the full length sequence; transforming recipient cells with the constructs, as described in Example 4; and screening the subsequently derived transgenic plants for activity, as described in Examples 5 and 6 of the specification. These teaching fully demonstrate the enablement of the claims.

3. Creation of Promoter Fragments is Routine in the Art

It must further be noted that it is well known that full length promoter fragments can be substantially deleted and mutated while still retaining promoter activity. For example, Cho and Cosgrove (2002) (*Plant Cell*, 14, 3237–3253; Section IX, **Exhibit A**) showed that more than 990 base pairs of an approximately 1428 bp plant promoter sequence designated AtEXP7 could be deleted without significantly effecting promoter activity and even larger deletions could be made while maintaining a reduced promoter activity. See **Exhibit A**, p. 3244, 2nd col. and FIG. 8. It was also shown that a deletion of approximately 775 bp could be made from a plant promoter designated AtEXP18 without significantly reducing promoter activity. See FIG. 10. The authors further showed that numerous substitution mutations could be made in a fragment of AtEXP7 retaining full activity while retaining promoter activity and in some cases increasing activity.

See. FIG. 9 and p. 3245, 2nd col. These studies therefore show that fragments of full length promoter sequences can routinely be made that retain promoter activity.

4. The Examiner Has Failed to Establish a *Prima Facie* Case of Lack of Enablement

The Examiner has provided no basis to doubt the enablement of the instant claims. Rather, the Action merely states that the specification does not provide sufficient structural and functional information regarding the claimed sequences for one of skill in the art to predict which species of SEQ ID NO:18 would have promoter activity without any objective basis for so concluding. However, it is the PTO that bears the burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by a claim is not adequately enabled by the description of the invention in the specification. *In re Wright*, 9 U.S.P.Q.2d 1510, 1512-1513 (Fed. Cir. 1993) (citing *In re Marzocchi*, 169 U.S.P.Q. 367, 369-70 (CCPA 1971)). Further, the evidence presented above demonstrates that one of skill in the art in view of the teaching in the specification could readily have made the claimed sequences without undue experimentation. A statement doubting the enablement of an Applicants' claims without providing an objective basis does not meet this standard, "[o]therwise, there would be no need for the Appellant to go to the trouble and expense of supporting his presumptively accurate disclosure." *In re Marzocchi*, 169 U.S.P.Q. at 370. Thus, without more, the rejection must fail.

It is finally noted that the legal standard for enablement does not require that Applicants demonstrate enablement for all possible claimed iterations. Enablement must bear only a reasonable relationship to the scope of the claims. *In re Fisher*, 166 U.S.P.Q. 18, 24 (CCPA 1970). For example, a patent applicant is not required to "predict every possible variation, improvement or commercial embodiment of his invention." *United States Steel Corp. v. Phillips Petroleum Co.*, 673 F. Supp. 1278, 1292 (D. Del. 1987), *aff'd,* 865 F.2d 1247, 1250 (Fed. Cir.

1989) (specifically quoting this statement). This is echoed in the MPEP: "[a]s long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied." MPEP 2164.01(b) (citing *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (CCPA 1970)).

In view of the foregoing, Applicants respectfully submit that the full scope of the claims has been enabled. Reversal of the rejection under 35 U.S.C. §112, first paragraph for lack of enablement is thus respectfully requested.

D. The Claims are not anticipated under 35 U.S.C. 102(e)

1. The Rejection Over Muhitch *et al.*

The Examiner rejects claims 1, 11-13, 15, 17-18, 20, 22, 24-25, 28-30, 34-37, 57-66, 69-70, and 72 under 25 U.S.C. § 102(e) as allegedly anticipated by Muhitch (U.S. Patent Pub. No. US20040148651 and WO01/92465). In particular, the Advisory Action indicates that it is the position of the Examiner that the reference teaches a 731 bp sequence that has a 258 bp portion with 98% identity to SEQ ID NO:18. In response, Applicants note that the current claims require a sequence comprising from "400 to 2547 contiguous nucleotides that hybridizes to the nucleic acid sequence of SEQ ID NO:18" under the recited high stringency wash conditions. The claims do not permit merely a 258 bp portion that hybridizes. A further review of the remaining portion of the recited sequence flanking the 258 bp portion in fact reveals numerous sequence differences. For example, as seen in the Examiner's search results of April 28, 2005 at pages 3-4 (Section IX, **Exhibit B**), upstream of a region of sequence similarity that apparently starts around base 474 and ends at base 731 (numbered according to the Muhitch WO01/92465 Figure 1 sequence found in the search results) SEQ ID NO:18 of the present application and the Muhitch

sequences do not share significant similarity. The sequences from Figure 1 of WO 01/92465 and SEQ ID NO:1 of U.S. 20040148651 contain a region of approximately 190-258 nucleotides that share about 98% similarity with SEQ ID NO:18 of Hinchey *et al.*

When looking at even the most identical longer stretches of any identity between Muhitch SEQ ID NO:1 and SEQ ID NO:18, far more differences are observed than would permit hybridization under stringent conditions. For example, aligning these sequences using the publicly available LALIGN program (http://www.ch.embnet.org/cgi-bin/LALIGN_form_parser) reveals that the most identical stretch even approaching 400 nucleotides, which involves a 353 nucleotide overlap corresponding to nucleotides 324-664 of Muhitch's SEQ ID NO:1, has only 77.1% identity. This is far below the degree of identity required for hybridization under high stringency conditions. Aligning a 400 bp segment yields even more significant differences. The Muhitch sequence therefore does not contain "400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18" or "400 to 2547 contiguous nucleotides that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes." As all elements of the claim are not in the prior art, the rejection is without basis.

Reversal of the rejection is thus respectfully requested.

2. The Rejection Over La Rosa *et al.*

The Advisory Action mailed by the Examiner April 10, 2006 indicates that this rejection has been withdrawn and thus the rejection is now moot.

E. <u>The Claims are Non-Obvious under 35 U.S.C. § 103</u>

The Examiner rejects claims 1 and 11-89 under 35 U.S.C. § 103(a) as obvious over Muhitch in combination with Wong *et al.* (1992), McCabe *et al.* (1988) and Poehlman and Sleper (1995). In response, Applicants note that none of the references teach or suggest the

claimed nucleic acids. Specifically, as explained above, the current claims require a sequence comprising from "400 to 2547 contiguous nucleotides that hybridizes to the nucleic acid sequence of SEQ ID NO:18" under the recited high stringency wash conditions. The claims do not permit merely a 258 bp portion that hybridizes.

As shown in **Exhibit B**, SEQ ID NO:18 of the present application and the Muhitch sequences do not share significant similarity absent the 258 bp portion. For example, when looking at even the most identical portions with any degree of identity between Muhitch SEQ ID NO:1 and current SEQ ID NO:18, substantial differences are observed than would not permit hybridization under stringent conditions. In particular, aligning these sequences using the publicly available LALIGN program (http://www.ch.embnet.org/cgi-bin/LALIGN_form_parser) reveals that the most identical stretch even approaching 400 nucleotides in length involves a 353 nucleotide overlap corresponding to nucleotides 324-664 of Muhitch's SEQ ID NO:1 that has only 77.1% identity. This is far below the degree of identity required for hybridization under high stringency conditions. Aligning a 400 bp segment yields would yield an even more significant difference.

The cited references or prior art generally therefore lack all elements of the claims. There further is simply no teaching or motivation to modify any sequence in the prior art such that it could correspond to the claimed sequences. The claims cannot therefore be deemed obvious under §103 and reversal of the rejection is thus respectfully requested.

CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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Date: May 22, 2006

VIII. CLAIMS APPENDIX

APPEALED CLAIMS:

1. (Previously presented) An isolated nucleic acid sequence comprising a cytoplasmic glutamine synthetase GS_{1-2} promoter, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises:

   (a) a nucleic acid sequence of SEQ ID NO:18 or a fragment thereof, having promoter activity, wherein the fragment comprises from 400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18; or

   (b) a nucleic acid sequence comprising from 400 to 2547 contiguous nucleotides that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes.

2-3. (Cancelled)

4. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 750 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

5. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 1000 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

6. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 1500 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

7. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 1750 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

8. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 2000 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

9. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 2250 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

10. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises from 2500 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18.

11. (Original) The isolated nucleic acid of claim 1, wherein the cytoplasmic glutamine synthetase GS_{1-2} promoter comprises the nucleic acid sequence of SEQ ID NO:18, or a fragment thereof comprising promoter activity.

12. (Original) The isolated nucleic acid of claim 1, further comprising an enhancer.

13. (Previously presented) The isolated nucleic acid of claim 12, wherein the enhancer comprises an intron.

14. (Original) The isolated nucleic acid of claim 13, wherein the intron is selected from the group consisting of the rice actin 1 intron and the rice actin 2 intron.

15. (Original) The isolated nucleic acid of claim 1, further comprising a 3' UTR.

16. (Original) The isolated nucleic acid of claim 15, wherein the 3' UTR comprises a *PIN II* 3' UTR.

17. (Previously presented) A transgenic plant stably transformed with a selected DNA comprising the cytoplasmic glutamine synthetase GS_{1-2} promoter of claim 1 operably linked to a selected heterologous coding region.

18. (Original) The transgenic plant of claim 17, wherein the selected heterologous coding region encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

19. (Original) The transgenic plant of claim 18, wherein the selected heterologous coding region encodes a protein imparting a selectable marker phenotype, wherein the protein is selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase, anthranilate synthase and glyphosate oxidoreductase.

20. (Original) The transgenic plant of claim 17, wherein the selected heterologous coding region is operably linked to a 3' UTR.

21. (Original) The transgenic plant of claim 20, wherein the 3' UTR is a *pinII* 3' UTR.

22. (Original) The transgenic plant of claim 17, wherein the selected DNA comprises an enhancer.

23. (Original) The transgenic plant of claim 22, wherein the enhancer is selected from the group consisting of rice actin 1 intron and rice actin 2 intron.

24. (Original) The transgenic plant of claim 17, wherein the selected DNA comprises plasmid DNA.

25. (Original) The transgenic plant of claim 17, wherein the selected DNA comprises a sequence encoding a signal peptide.

26. (Original) The transgenic plant of claim 25, wherein the signal peptide comprises a chloroplast transit peptide.

27. (Original) The transgenic plant of claim 17, comprising a sequence encoding a transit peptide, wherein the transit peptide is selected from the group consisting of chlorophyll a/b binding protein transit peptide, small subunit of ribulose bisphosphate carboxylase transit peptide, EPSPS transit peptide and dihydrodipocolinic acid synthase transit peptide.

28. (Original) The transgenic plant of claim 17, further defined as a monocotyledonous plant.

29. (Original) The transgenic plant of claim 28, wherein the monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane.

30. (Original) The transgenic plant of claim 29, wherein the monocotyledonous plant is maize.

31. (Original) The transgenic plant of claim 17, further defined as a dicotyledonous plant.

32. (Original) The transgenic plant of claim 31, wherein the dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton.

33. (Original) The transgenic plant of claim 32, wherein the dicotyledonous plant is a soybean plant.

34. (Original) The transgenic plant of claim 17, further defined as a fertile R_0 transgenic plant.

35. (Original) A seed of the fertile R_0 transgenic plant of claim 34, wherein the seed comprises the selected DNA.

36. (Original) The transgenic plant of the claim 17, further defined as a progeny plant of any generation of a fertile R_0 transgenic plant.

37. (Original) A seed of the progeny plant of claim 36, wherein the seed comprises the selected DNA.

38. (Previously presented) A crossed fertile transgenic plant prepared according to the method comprising the steps of:

(i) obtaining a fertile transgenic plant comprising a selected DNA comprising the cytoplasmic glutamine synthetase GS_{1-2} promoter of claim 1;

(ii) crossing the fertile transgenic plant with itself or with a second plant to prepare the seed of a crossed fertile transgenic plant, wherein the seed comprises the selected DNA; and

(iii) planting the seed to obtain a crossed fertile transgenic plant.

39. (Original) The crossed fertile transgenic plant of claim 38, wherein the second plant lacks the selected DNA.

40. (Original) A seed of the crossed fertile transgenic plant of claim 38, wherein the seed comprises the selected DNA.

41. (Original) The crossed fertile transgenic plant of claim 38, further defined as a monocotyledonous plant.

42. (Original) The crossed fertile transgenic plant of claim 41, wherein the monocotyledonous plant is selected from the group consisting of wheat, oat, barley, maize, rye, rice, turfgrass, sorghum, millet and sugarcane.

43. (Original) The crossed fertile transgenic plant of claim 42, wherein the monocotyledonous plant is a maize plant.

44. (Original) The crossed fertile transgenic plant of claim 38, further defined as a dicotyledonous plant.

45. (Original) The crossed fertile transgenic plant of claim 44, wherein the dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, alfalfa, sunflower and cotton.

46. (Original) The crossed fertile transgenic plant of claim 45, wherein the dicotyledonous plant is a soybean plant.

47. (Original) The crossed fertile transgenic plant of claim 38, wherein the selected DNA is inherited through a female parent.

48. (Original) The crossed fertile transgenic plant of claim 38, wherein the selected DNA is inherited through a male parent.

49. (Original) The crossed fertile transgenic plant of claim 38, wherein the second plant is an inbred plant.

50. (Original) The crossed fertile transgenic plant of claim 49, wherein the crossed fertile transgenic plant is a hybrid.

51. (Original) The crossed fertile transgenic plant of claim 38, wherein the selected DNA comprises a selected heterologous coding region operably linked to the maize cytoplasmic glutamine synthetase GS_{1-2} promoter.

52. (Original) The crossed fertile transgenic plant of claim 51, wherein the selected coding region encodes a protein selected from the group consisting of a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

53. (Original) The crossed fertile transgenic plant of claim 38, wherein the selected DNA comprises an enhancer.

54. (Original) The crossed fertile transgenic plant of claim 53, wherein the enhancer is selected from the group consisting of rice actin 1 intron and rice actin 2 intron.

55. (Original) The crossed fertile transgenic plant of claim 51, wherein the selected coding region is operably linked to a 3' UTR.

56. (Original) The crossed fertile transgenic plant of claim 55, wherein the 3' UTR is a *pinII* 3' UTR.

57. (Previously presented) A method of preparing a transgenic plant comprising the steps of:

(i) obtaining a construct comprising the cytoplasmic glutamine synthetase GS_{1-2} promoter of claim 1;

(ii) transforming a recipient plant cell with the construct; and

(iii) regenerating the recipient plant cell to obtain a transgenic plant transformed with the construct.

58. (Original) The method of claim 57, wherein the maize cytoplasmic glutamine synthetase GS_{1-2} promoter is operably linked to a selected coding region.

59. (Original) The method of claim 57, wherein the transgenic plant is fertile.

60. (Original) The method of claim 59, further comprising the step of obtaining seed from the fertile transgenic plant.

61. (Original) The method of claim 60, further comprising obtaining a progeny plant of any generation from the fertile transgenic plant.

62. (Original) The method of claim 57, wherein the step of transforming comprises a method selected from the group consisting of microprojectile bombardment, PEG mediated transformation of protoplasts, electroporation, silicon carbide fiber mediated transformation, or *Agrobacterium*-mediated transformation.

63. (Original) The method of claim 62, wherein the step of transforming comprises microprojectile bombardment.

64. (Original) The method of claim 57, wherein the recipient plant cell is from a monocotyledonous plant.

65. (Original) The method of claim 64, wherein the monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, turfgrass, oat, barley, sorghum, millet, and sugarcane.

66. (Original) The method of claim 65, wherein the monocotyledonous plant is a maize plant.

67. (Original) The method of claim 57, wherein the recipient plant cell is from a dicotyledonous plant.

68. (Original) The method of claim 67, wherein the dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

69. (Original) The method of claim 58, wherein the selected coding region encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

70. (Original) The method of claim 57, wherein the construct comprises an enhancer.

71. (Original) The method of claim 70, wherein the enhancer is selected from the group consisting of rice actin 1 intron and rice actin 2 intron.

72. (Original) The method of claim 58, wherein the selected coding region is operably linked to a 3' UTR.

73. (Original) The method of claim 72, wherein the 3' UTR is a *pinII* 3' UTR.

74. (Previously presented) A method of plant breeding comprising the steps of:

(i) obtaining a transgenic plant comprising a selected DNA comprising the cytoplasmic glutamine synthetase GS_{1-2} promoter of claim 1; and

(ii) crossing the transgenic plant with itself or a second plant.

75. (Original) The method of claim 74, wherein the transgenic plant is a monocotyledonous plant.

76. (Original) The method of claim 75, wherein the monocotyledonous plant is selected from the group consisting of wheat, maize, oat, barley, rye, rice, turfgrass, sorghum, millet and sugarcane.

77. (Original) The method of claim 76, wherein the monocotyledonous plant is a maize plant.

78. (Original) The method of claim 74, wherein the transgenic plant is a dicotyledonous plant.

79. (Original) The method of claim 78, wherein the dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

80. (Original) The method of claim 74, wherein the transgenic plant is crossed with the second plant.

81. (Original) The method of claim 80, wherein the second plant is an inbred plant.

82. (Original) The method of claim 74, further comprising the steps of:

(iii) collecting seeds resulting from the crossing;

(iv) growing the seeds to produce progeny plants;

(v) identifying a progeny plant comprising the selected DNA; and

(vi) crossing the progeny plant with itself or a third plant.

83. (Original) The method of claim 82, wherein the progeny plant inherits the selected DNA through a female parent.

84. (Original) The method of claim 82, wherein the progeny plant inherits the selected DNA through a male parent.

85. (Original) The method of claim 82, wherein the second plant and the third plant are of the same genotype.

86. (Original) The method of claim 85, wherein the second and third plants are inbred.

87. (Original) The method of claim 74, wherein the selected DNA further comprises a coding region, wherein the coding region encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

88. (Original) The method of claim 74, wherein the selected DNA further comprises a genetic element which enhances the expression of the protein in the transgenic plant.

89. (Original) The method of claim 88, wherein the genetic element is selected from the group consisting of the rice actin 1 intron and the rice actin 2 intron.

IX. EVIDENCE APPENDIX

Exhibit A: Cho *et al.,* 2002. Plant Cell 14:3237-3253; cited, for example, in Response to Official Action filed September 20, 2005, page 20.

Exhibit B: Pages 3-4 excerpt of Examiner's Search Results of April 28, 2005, found at USPTO Private Pair website for U.S. Patent Application 09/989,739. Cited in Examiner's Final Rejection dated December 16, 2005, page 6, line 7.

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Regulation of Root Hair Initiation and Expansin Gene Expression in Arabidopsis[W]

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The expression of two Arabidopsis expansin genes (*AtEXP7* and *AtEXP18*) is tightly linked to root hair initiation; thus, the regulation of these genes was studied to elucidate how developmental, hormonal, and environmental factors orchestrate root hair formation. Exogenous ethylene and auxin, as well as separation of the root from the medium, stimulated root hair formation and the expression of these expansin genes. The effects of exogenous auxin and root separation on root hair formation required the ethylene signaling pathway. By contrast, blocking the endogenous ethylene pathway, either by genetic mutations or by a chemical inhibitor, did not affect normal root hair formation and expansin gene expression. These results indicate that the normal developmental pathway for root hair formation (i.e., not induced by external stimuli) is independent of the ethylene pathway. Promoter analyses of the expansin genes show that the same promoter elements that determine cell specificity also determine inducibility by ethylene, auxin, and root separation. Our study suggests that two distinctive signaling pathways, one developmental and the other environmental/hormonal, converge to modulate the initiation of the root hair and the expression of its specific expansin gene set.

INTRODUCTION

Root hairs are polarized outgrowths of root epidermal cells. In Arabidopsis, root hairs normally arise from epidermal cells that contact two underlying cortical cells (the so-called H position), whereas epidermal cells overlying a single cortical cell (in the N position) develop into nonhair cells (Dolan et al., 1993; Galway et al., 1994). This position-dependent hair cell differentiation thus results in a striped pattern of hair cell files along the long axis of the root, which is found in members of Brassicaceae and in a few species of other families (Cormack, 1947; Dolan and Costa, 2001). Root hair development in Arabidopsis can be divided into three phases: cell specification, initiation, and elongation. Cell specification refers to the fate determination of epidermal cells into hair cells and nonhair cells, depending on position. Initiation refers to the formation of a protrusion or bulge at the site of hair outgrowth. Elongation refers to the process of sustained tip growth that normally follows initiation. Numerous experimental observations indicate that these three phases involve different cellular and genetic processes (for reviews, see Schiefelbein, 2000; Foreman and Dolan, 2001).

Several genes that control root epidermal cell specification have been identified. Loss-of-function mutations in TTG (TRANSPARENT TESTA/GLABROUS) or GL2 (GLABRA2) result in root hairs in both H and N positions (Galway et al., 1994; Masucci et al., 1996), indicating that TTG (a protein with WD40 repeats) and GL2 (a homeodomain transcription factor) function as negative regulators of the differentiation of nonhair cells to hair cells. Mutations in another MYB transcription factor, WER (WEREWOLF), also generate root hairs in almost every root epidermal cell, because WER positively regulates GL2 expression (Lee and Schiefelbein, 1999). On the other hand, *cpc* (*caprice*) mutants have only a few root hairs, indicating that CPC, a MYB-like protein, functions as a positive regulator for root hair cell differentiation (Wada et al., 1997). A recent study demonstrated the interactions among these regulatory genes (Lee and Schiefelbein, 2002). In the N position, WER positively regulates the expression of CPC and GL2. CPC (or its downstream signal) appears to move to cells in the H position and inhibits the expression of WER, CPC, and GL2, which leads the cell to initiate hair formation.

Root hair initiation, which is genetically downstream of GL2 (Masucci and Schiefelbein, 1996), is regulated by another set of genes and is sensitive to hormonal and environmental factors (Schiefelbein, 2000). The auxin-resistant mutant (*axr2*) develops few root hair bulges (Wilson et al., 1990), and the defect of root hair initiation in root hair defective (*rhd6*) can be reversed by treatment with auxin or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid

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[W]Online version contains Web-only data.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.006437.

(ACC) (Masucci and Schiefelbein, 1994, 1996). ACC treatment of wild-type plants induces root hairs in the N position, as do the constitutively ethylene-responsive *ctr1* and ethylene-overproducing *eto* mutants (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999). ACC has been suggested as a factor that determines the developmental fate of cells in the H position (Tanimoto et al., 1995). Also implicating ethylene involvement in root hair initiation, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) and silver ion (an inhibitor of ethylene perception) have been found to inhibit root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). However, the role of ethylene in root hair formation is questioned because the ethylene-insensitive mutants *etr1* and *ein2* maintained normal root hair numbers (Masucci and Schiefelbein, 1996). Additionally, environmental factors such as nutrients (Peterson and Stevens, 2000), light, and separation of the root from the agar medium (Okada and Shimura, 1994) also affect root hair development. It has been suggested that hormones and environmental factors affect root hair initiation through a pathway distinctive from the normal development-associated pathway (Okada and Shimura, 1994; Schiefelbein, 2000), but experimental confirmation for this is needed.

Elongation of the root hair is achieved by tip growth (Schiefelbein, 2000). Hair elongation likely is governed by genetic components distinct from those that govern hair initiation, but root hair elongation is influenced by auxin, ethylene, and environmental factors as well (Okada and Shimura, 1994; Pitts et al., 1998; Schiefelbein, 2000).

Spatial regulation of cell wall expansion is critical for cell morphogenesis in plants (Fowler and Quatrano, 1997). Thus, outgrowth of the root hair from the epidermal cell is expected to accompany localized cell wall loosening at the correct position. Bibikova et al. (1998) demonstrated localized wall acidification at the site of root hair initiation. This acidification could activate expansins. Expansins are cell wall–loosening proteins capable of mediating cell wall extension in acidic conditions without hydrolytic breakage of major structural components of the cell wall (McQueen-Mason et al., 1992; for recent reviews, see Cosgrove, 2000; Lee et al., 2001). Expansin genes are found throughout the entire plant kingdom (Cosgrove, 1999; Li et al., 2002), and their pattern of expression indicates that they are related closely to cell growth and tissue differentiation (for review, see Cho, 2001). Alteration of endogenous expansin gene expression modulates leaf growth and pedicel abscission in Arabidopsis (Cho and Cosgrove, 2000) and leaf morphology and phylotaxy in tobacco (Pien et al., 2001). Two families of expansins are recognized at present (Cosgrove, 2000), α- and β-expansins, and Arabidopsis has 26 α- and 5 β-expansin genes (see http://www.bio.psu.edu/expansins). In the course of analyzing the expression of these genes in Arabidopsis, two α-expansin genes, *AtEXP7* and *AtEXP18*, were found to be expressed specifically in root hair cells (D.M. Durachko and D.J. Cosgrove, unpublished data).

In this study, we examined in detail the expression patterns of these two root hair–specific expansin genes in various root hair mutants as well as under hormonal (auxin and ethylene) and environmental (separation of the root from the medium) treatments. In particular, the role of endogenous ethylene in root hair development was studied closely. Promoter analyses of the two expansin genes, in conjunction with the effect of root hair–inducing factors, also were conducted to elucidate the regulation of expression of these root hair–specific genes. Our results show that the expression of these expansin genes is linked tightly to root hair initiation and subsequent elongation. Moreover, we find that, although ethylene mediates the effects of auxin and root separation on root hair initiation, it is not essential for the normal (or default) development of root hairs in wild-type plants. These results alter current views of ethylene involvement in root hair development.

RESULTS

Root Hair Cell–Specific Expression of *AtEXP7* and *AtEXP18*

RNA gel blot and promoter-reporter gene expression analyses were performed to investigate the organ- and tissue-specific expression patterns of *AtEXP7* and *AtEXP18*. The transcripts of both expansin genes were found in the root but were undetectable in other major plant organs (Figure 1). Wild-type plants harboring the *AtEXP7* promoter::β-glucuronidase (*GUS*) or *AtEXP7* promoter::green fluorescent protein (*GFP*) construct showed staining (or fluorescence) solely

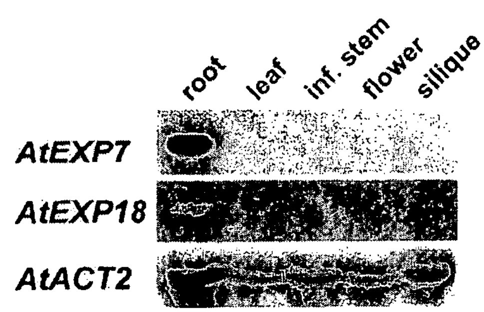

Figure 1. Expression of *AtEXP7* and *AtEXP18* in Different Tissues.

Total RNA was isolated from seedling roots, young leaves, growing inflorescence (inf.) stems, whole floral organs, and young green siliques of Columbia wild-type Arabidopsis plants. Twenty micrograms of total RNA was analyzed per lane. The transcript levels of Arabidopsis actin2 (*AtACT2*) served as a loading control.

in root hair cell files (Figures 2A to 2D). No reporter gene expression was found in other cell types of the root or other organs except a weak expression in the inner layer of the seed coat. Different ecotypes, Columbia and Wassilewskija, showed the same reporter gene expression pattern. The expression of *AtEXP7* occurred approximately one cell before the root hair bulges appeared (Figure 2B), indicating the gene's close temporal expression with the hair initiation process. Plants harboring the *AtEXP18* promoter::reporter construct also showed the same expression pattern as plants with the *AtEXP7* promoter::reporter construct (data not shown). However, the level of *AtEXP18* expression was lower than that of *AtEXP7*. Promoter analyses, as described below, showed that the average promoter activity of *AtEXP18* was ~60% of *AtEXP7* promoter activity. In this study, the expression pattern of *AtEXP7* is described in greater detail, but the results also hold for *AtEXP18*.

The AtEXP7 protein expression pattern also was examined by expressing the AtEXP7-GFP fusion protein driven by the *AtEXP7* promoter. The cell-type specificity and the timing of protein expression were almost identical with the expression pattern of the reporter gene alone (Figures 2E to 2G). The fluorescence from the fusion protein was highest in regions of root hair initiation and elongation. Although the AtEXP7-GFP fusion protein tended to localize more at the emerging root hair tip and to distribute peripherally in the root hair cell (Figures 2E to 2G), it was detected predominantly inside the plasma membrane upon plasmolysis (data not shown). This finding indicates that the fusion protein was not secreted to the cell wall.

We have searched for mutants defective in *AtEXP7* or *AtEXP18*. An Arabidopsis line that includes a T-DNA insertion in the second intron of *AtEXP7* was identified, but the homozygous line still expressed transcripts of the correct size, albeit at a lower level than in the wild type. This line did not show obvious alterations in the root hair, most likely as a result of the leakiness of the mutation and functional redundancy by *AtEXP18* and perhaps other expansin genes.

Effect of Root Hair–Regulating Factors on the Expression of Root Hair Expansin Genes

Root hair formation in Arabidopsis is regulated by developmental regulators, hormones, and environmental factors. Because *AtEXP7* is a root hair–specific gene and is thought to function in root hair formation, we investigated whether *AtEXP7* expression is modulated by various root hair–regulating factors. For this purpose, the *AtEXP7* promoter::*GUS* reporter construct was introduced into root hair mutants, and the reporter gene expression pattern was monitored.

In *ttg* and *gl2* mutants, which have hairs in both the H and N positions, *AtEXP7* promoter::*GUS* was expressed in both positions (Figures 2H and 2I), suggesting that TTG and GL2 negatively regulate the expression of *AtEXP7*, just as they negatively regulate root hair formation in the N position of the wild-type plant (Galway et al., 1994; Masucci et al., 1996).

The *axr2* mutant is defective in hair elongation and partially in hair initiation; thus, it produces few root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2J). The spatial pattern of *AtEXP7* promoter::*GUS* expression was not changed in this mutant (Figure 2J) compared with that in wild-type plants. However, the expression level of *AtEXP7* was much lower in the mutant than in the wild type (Figure 3). Because auxin positively regulates root hair formation and *AtEXP7* expression (see below), AXR2 likely downregulates the expression of *AtEXP7* and partially inhibits root hair formation.

The mutant *rhd6* also is defective in root hair initiation, but unlike *axr2*, it develops almost no root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2K). *AtEXP7* expression in *rhd6* was blocked almost completely, as shown by GUS expression and by transcript analysis (Figures 2K and 3). In *rhd6*, treatment with the ethylene precursor ACC or auxin, or separation of the root from the agar medium induced normal root hair formation (Masucci and Schiefelbein, 1994, 1996) (Table 1). In agreement with their effects on root hair formation, all of these treatments induced *AtEXP7* expression in *rhd6* roots (Figures 2L to 2N and 3). *AtEXP18* expression in *rhd6*, as described below, also was inducible by these treatments. These results indicate that RHD6 is a positive regulator of *AtEXP7* and *AtEXP18* expression.

Exogenous Ethylene Is a Positive Effector for the Expression of Root Hair Expansin Genes in Concert with Root Hair Formation

Because ethylene is a positive effector of root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995), we investigated whether ethylene coordinately regulates the expression of root hair expansin genes with root hair formation. The ethylene precursor ACC (5 μM) induced root hair formation and *AtEXP7* expression in the N position of the wild-type root (Figure 2O). Mutation in CTR1, which showed constitutive ethylene effects and thus induced the formation of root hairs in the N position (Table 1), likewise activated *AtEXP7* expression in root hairs in the N position (Figure 2R) and increased the transcript level by 36% relative to that of the wild type (Figure 3). Compared with the wild type, the root hair–defective *rhd6* mutant had only ~10% of the *AtEXP7* transcript (Figure 3), which could derive from the occasional root hairs in the *rhd6* root. Treatment of the mutant with 5 μM ACC restored 78% of the transcript level and 74% of the root hair number (Table 1). Ethylene gas (1 μL/L) treatment also induced a similar level of root hairs in the *rhd6* root, as did 5 μM ACC (data not shown), and the effect of exogenous ethylene or ACC could be blocked completely by 1-methylcyclopropene (1-MCP), the competitive inhibitor of ethylene binding to the

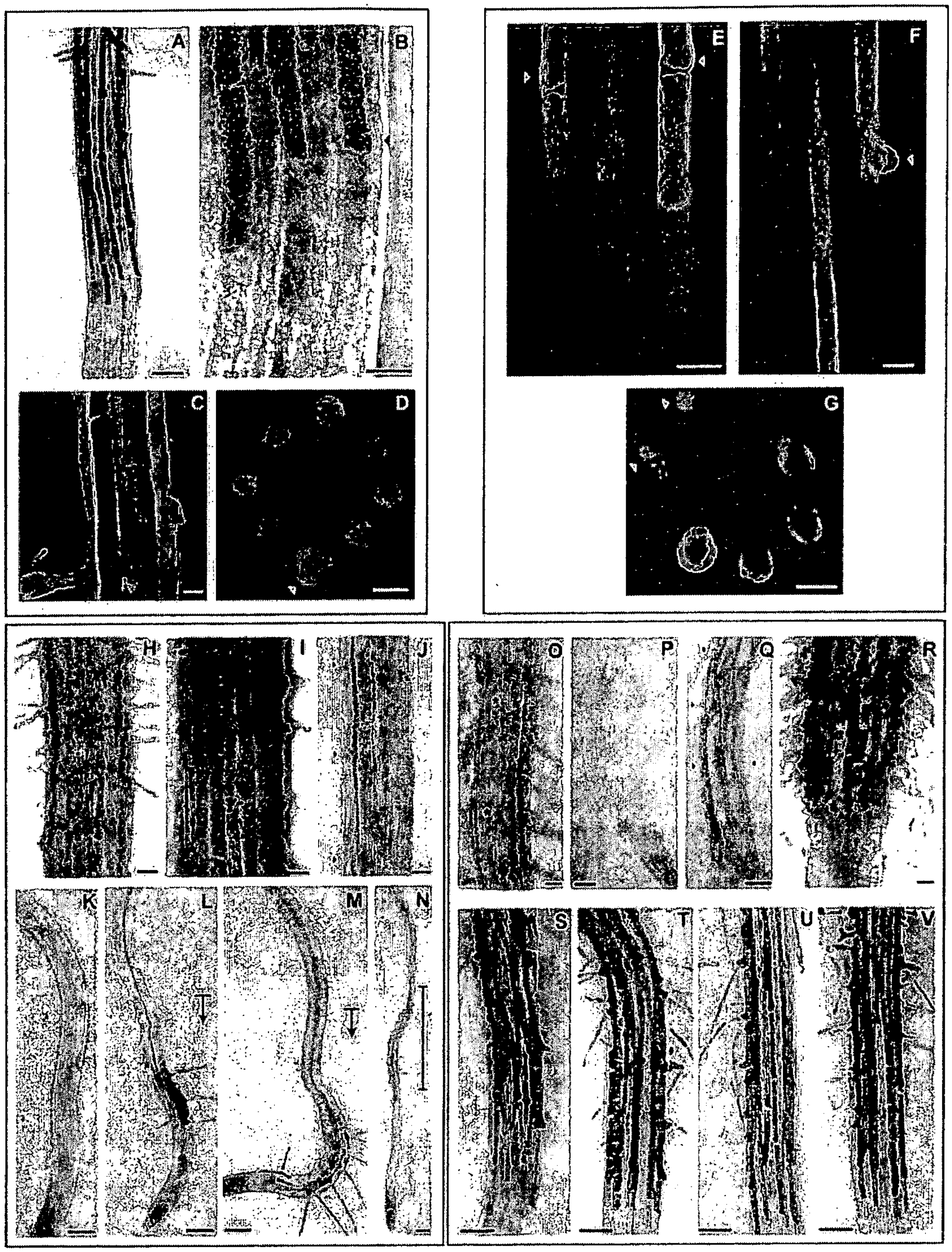

Figure 2. Root Hair Cell–Specific Expression Pattern of *AtEXP7* in the Arabidopsis Root.

(A), **(B)**, and **(H)** to **(V)** show *AtEXP7* promoter::*GUS* expression; **(C)** and **(D)** show *AtEXP7* promoter::*GFP* expression; and **(E)** to **(G)** show *AtEXP7* promoter::genomic *AtEXP7-GFP* expression.

(A) to **(D)** In the wild-type root, reporter gene expression occurs in the root hair cell files. The weaker blue staining between the strong stains are from the hair cell files of the opposite side. **(C)** shows an optical longitudinal section demonstrating GFP expression at the root hair cell files. The

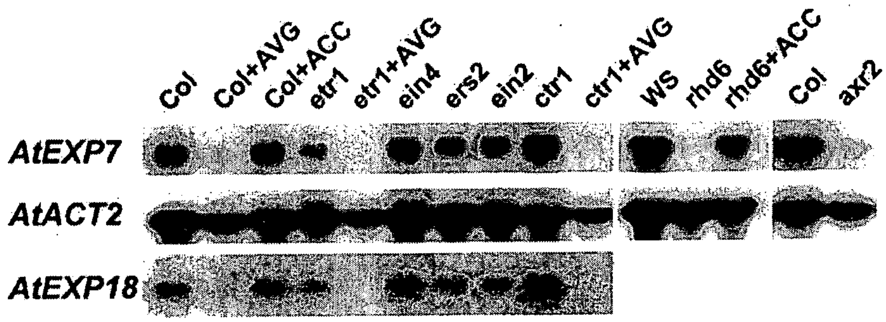

Figure 3. RNA Gel Blot Analyses of *AtEXP7* and *AtEXP18* Transcripts in Different Mutant Backgrounds and under Treatment with Ethylene Precursor and Inhibitor.

Total RNA was prepared from roots of 4-day-old wild-type and mutant seedlings. For ACC (5 μM) and AVG (5 μM) treatments, the seedlings were transferred to chemical-containing plates on day 3. Ten micrograms of total RNA, except for Wassilewskija and *rhd6* (30 μg), was analyzed. The transcript level of Arabidopsis actin2 (*AtACT2*) served as a loading control. Col, Columbia wild type; WS, Wassilewskija wild type.

receptors. We chose 1-MCP as an antagonist of ethylene action because of its high specificity of action and lack of deleterious side effects (Sisler et al., 1996; Hall et al., 2000). At 1 μL/L, 1-MCP almost completely abolished ACC-induced root hair formation and the expression of *AtEXP7* and *AtEXP18* in *rhd6* (Figure 4).

1-MCP Inhibits Auxin- or Root Separation–Induced Root Hair Formation and Expression of Root Hair Expansin Genes

To investigate the possible involvement of ethylene receptors in root hair formation and expansin gene expression induced by auxin or root separation from the agar medium, the antagonism of these effectors by 1-MCP was investigated in the *rhd6* background. Auxin- or root separation–induced root hair formation was greatly inhibited by 1-MCP (1 μL/L). No root hair bulges or elongated root hairs were observed in mutant seedlings treated with indole 3-acetic acid (IAA; 30 nM) together with 1-MCP (Figure 5B). Similarly, 1-MCP inhibited 90% of the root hair formation induced by root separation (Figures 6B and 6C). Consistent with these results, 1-MCP inhibited 70 to 90% of IAA- or root separation–induced expression of *AtEXP7* and *AtEXP18* (Figures 5C to 5H and 6D to 6I). These results show that the coordinate induction of root hairs and expansin gene expression by auxin and root separation requires ethylene sensing, most likely because ethylene is part of the signaling pathway for these effects.

Figure 2. (continued).

red area from propidium iodide indicates the cell boundary. **(D)** shows an optical cross-section of the root demonstrating gene expression at the eight root hair cells. The arrowheads in **(B)** and **(D)** indicate emerging root hair bulges.

(E) to **(G)** Expression of the AtEXP7-GFP fusion protein shows the same pattern as expression of GUS or GFP alone. **(G)** shows an optical cross-section. Arrowheads indicate emerging root hair bulges.

(H) and **(I)** In the *ttg-1* **(H)** and *gl2-1* **(I)** backgrounds, reporter gene expression is observed in cells from both the H and N positions.

(J) *axr2-1* background. Arrowheads indicate some root hair bulges.

(K) to **(N)** *rhd6* background with no treatment **(K)** or with 5 μM ACC **(L)**, 30 nM IAA **(M)**, or separation of the root from the medium **(N)**. The bases of the arrows in **(L)** and **(M)** indicate the approximate starting points of hormone treatments. The vertical bar in **(N)** indicates where the root was separated from agar.

(O) to **(Q)** Wild-type roots treated with 5 μM ACC **(O)**, 5 μM AVG **(P)**, or 50 μM silver ion **(Q)**. Stars in **(O)** indicate ectopic expression of GUS in the N positions.

(R) *ctr1-1* background. Stars indicate ectopic expression of GUS in the N positions.

(S) to **(U)** Dominant ethylene receptor mutants *etr1-1* **(S)**, *ein4* **(T)**, and *ers2-1* **(U)**.

(V) *ein2-1* background.

Bars = 100 μm in **(K)** to **(N)**, 50 μm in **(A)**, **(P)**, **(Q)**, and **(S)** to **(V)**, and 20 μm in **(B)** to **(J)**, **(O)**, and **(R)**.

Table 1. Root Hair Number in Wild-Type and Mutant Plants with Ethylene Precursor or Inhibitor Treatment

Plant	Percent of Total Root Hair Cells[a]				Percent of Root Hair Cells in the N position[b]	
	No Treatment	ACC (5 μM)	AVG (5 μM)	1-MCP (1 μL/L)	No Treatment	ACC (5 μM)
Columbia	51.1 ± 3.3	65.9 ± 5.8	1.3 ± 2.3	44.0 ± 4.2	1.1 ± 3.3	15.9 ± 5.8
etr1-1	45.8 ± 6.7	48.1 ± 3.7	0 ± 0	43.0 ± 2.7	2.7 ± 3.3	2.5 ± 4.6
etr2	51.3 ± 2.3	51.5 ± 3.4	0.6 ± 1.8	48.3 ± 2.5	1.3 ± 2.3	1.5 ± 3.4
ers1	55.4 ± 6.2	52.5 ± 4.2	0.6 ± 1.8	49.5 ± 2.7	5.4 ± 6.2	2.5 ± 4.2
ers2-1	50.0 ± 3.0	55.0 ± 8.2	0 ± 0	44.1 ± 3.8	0.8 ± 1.9	6.4 ± 8.5
ein4	50.4 ± 1.4	57.2 ± 8.7	0 ± 0	42.3 ± 3.5	0.4 ± 1.4	7.2 ± 8.7
ein2-1	48.8 ± 3.8	45.8 ± 2.0	0 ± 0	N.D.[c]	2.5 ± 3.4	0 ± 0
ctr1-1	65.0 ± 6.4	N.D.	0 ± 0	N.D.	15.0 ± 6.4	N.D.
eto2	63.8 ± 4.8	N.D.	0 ± 0	N.D.	13.8 ± 4.8	N.D.
etr1-7	50.7 ± 5.1	N.D.	N.D.	N.D.	0.7 ± 5.1	N.D.
rhd6	0 ± 0	37.6 ± 13.9	N.D.	N.D.	0 ± 0	3.7 ± 6.6

Values shown are means ± SD (n = 140 to 260).
[a] Percentage of root hair–bearing epidermal cells among total epidermal cells counted, including cells in both the H and N positions.
[b] Percentage of root hair–bearing epidermal cells at the N position among total epidermal cells counted.
[c] N.D., not determined.

Endogenous Ethylene Is Not Involved in Normal (Default) Root Hair Formation and Expression of Root Hair Expansin Genes in the Wild Type

To verify the role of endogenous ethylene during root hair formation and expression of root hair expansin genes, we examined the effects of dominant mutations of ethylene receptors and inhibitors of ethylene action. Here, we use the term "endogenous ethylene" to designate the internal ethylene level in the plant without any mutations or treatments that would induce the overproduction of ethylene.

Our results showed that mutations in the ethylene signaling pathway failed to inhibit root hair formation and expansin gene expression. None of the five dominant-negative ethylene receptor mutants showed a significant reduction in root hair density (Table 1). The *ein2* mutant, which is known to exhibit the strongest ethylene phenotype, also had a normal number of root hairs, consistent with a previous report (Masucci and Schiefelbein, 1996). *AtEXP7* expression also was patterned normally in roots of the ethylene mutants (Figures 2S to 2V), and expression levels were not reduced greatly in the mutant backgrounds (Figure 3).

Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, has been used to test the role of ethylene in root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). Our results showed that 5 μM AVG almost completely blocked root hair formation and *AtEXP7* expression in the wild type (Figures 2P and 3, Table 1). However, surprisingly, AVG (5 μM) almost completely inhibited root hair formation in the constitutively ethylene-responsive mutant *ctr1-1* (Table 1), even though this mutant should not respond to AVG inhibition of ethylene synthesis. AVG markedly repressed the expression of *AtEXP7* in *ctr1-1* and other genotypes, but it also reduced actin gene expression (*AtACT2*; Figure 3). Although it was reported that ACC could partially restore root hair formation in the AVG-treated root (Masucci and Schiefelbein, 1994, 1996), our results indicate that AVG has significant deleterious effects on root hair development. Toxicity of AVG also is reported in root formation (Jackson, 1991) and somatic embryogenesis (Meijer, 1989). This may occur because AVG, functioning as an inhibitor of pyridoxal phosphate–dependent enzymes (Abel, 1985), probably interferes with other biochemical processes that are vulnerable to the inhibitor, not only ethylene biosynthesis.

To further test the role of endogenous ethylene during root hair formation in wild-type plants, we used 1-MCP, which binds to multiple ethylene receptors (Hall et al., 2000). Thus, we expected that 1-MCP would strongly inhibit root hair formation in wild-type plants if endogenous ethylene were involved. However, root hair formation in the wild-type root was inhibited very little by 1 μL/L 1-MCP (Table 1) or even by 10 μL/L (data not shown). By contrast, 0.22 μL/L 1-MCP showed saturated inhibitory effects on both ethylene binding to the receptors and the triple response (Hall et al., 2000). 1-MCP also did not significantly inhibit the expression of *AtEXP7* and *AtEXP18* in the wild type (Figure 7). 1-MCP is not able to reverse the constitutive ethylene-responsive phenotype of *ctr1* (Hall et al., 2000), in contrast to the deleterious effect of AVG on the *ctr1* root.

Silver ion (an inhibitor of ethylene perception) at 50 μM did not abolish root hair formation and *AtEXP7* expression, although it completely inhibited hair elongation (Figure 2Q). A previous study reported that silver ion (1 μM) greatly reduced root hair number (Tanimoto et al., 1995),

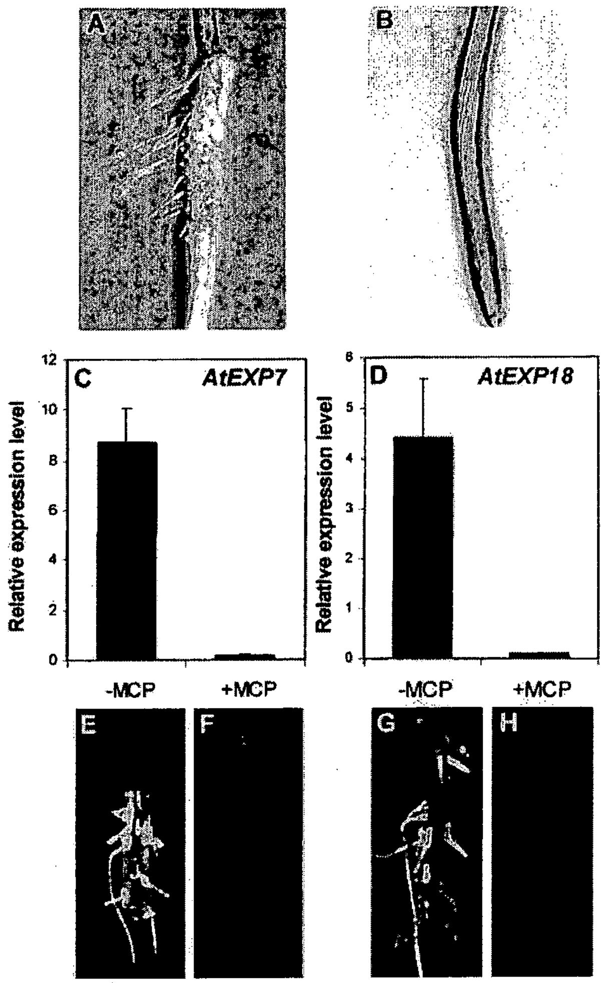

Figure 4. Effect of 1-MCP on ACC-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of roots grown in 5 μM ACC without **(A)** or with **(B)** 1 μL/L 1-MCP.
(C) and **(D)** Relative expression levels of *AtEXP7* **(C)** and *AtEXP18* **(D)** in the root when induced by 5 μM ACC without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 11 to 18).
(E) to **(H)** Confocal microscopy images of the roots harboring *AtEXP7* promoter::*GFP* (**[E]** and **[F]**) and *AtEXP18* promoter::*GFP* (**[G]** and **[H]**). Seedlings were incubated in 5 μM ACC without (**[E]** and **[G]**) or with (**[F]**) and **[H]**) 1-MCP.

but it is not clear whether small bulges were counted. The effects of ACC, AVG, and mutations in ethylene signaling on the expression of *AtEXP18* also resembled those on *AtEXP7* expression, as shown by RNA gel blot analysis (Figure 3).

Endogenous Ethylene Affects Root Hair Elongation

In contrast to root hair initiation, ethylene showed an unambiguous effect on root hair elongation, consistent with a

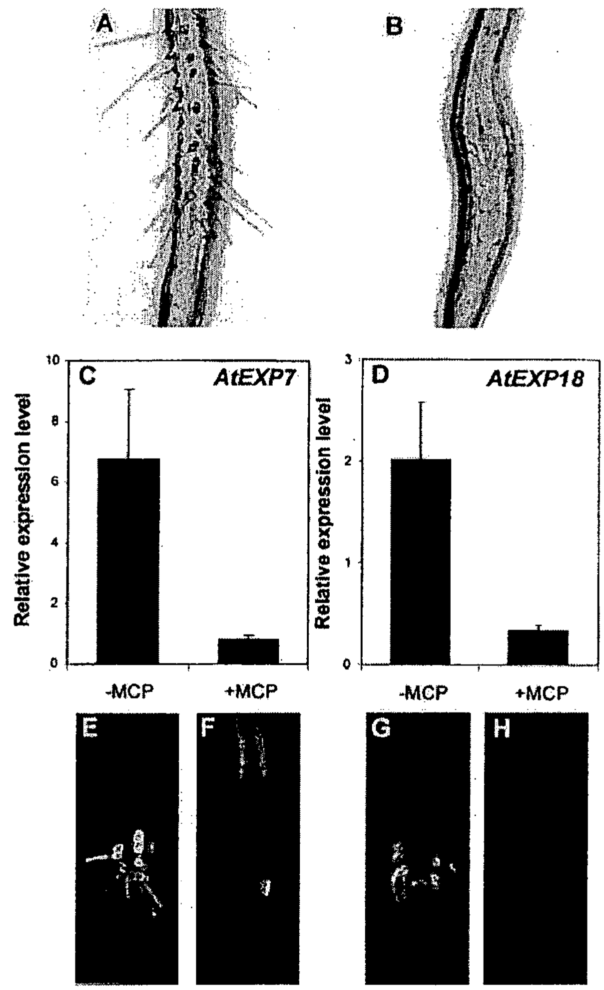

Figure 5. Effect of 1-MCP on IAA-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of the roots grown in 30 nM IAA without **(A)** or with **(B)** 1 μL/L 1-MCP.
(C) and **(D)** Relative expression levels of *AtEXP7* **(C)** and *AtEXP18* **(D)** in the root when induced by IAA without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 7 to 12).
(E) to **(H)** Confocal microscopy images of the roots harboring *AtEXP7* promoter::*GFP* (**[E]** and **[F]**) and *AtEXP18* promoter::*GFP* (**[G]** and **[H]**). Seedlings were incubated in IAA without (**[E]** and **[G]**) or with (**[F]** and **[H]**) 1-MCP.

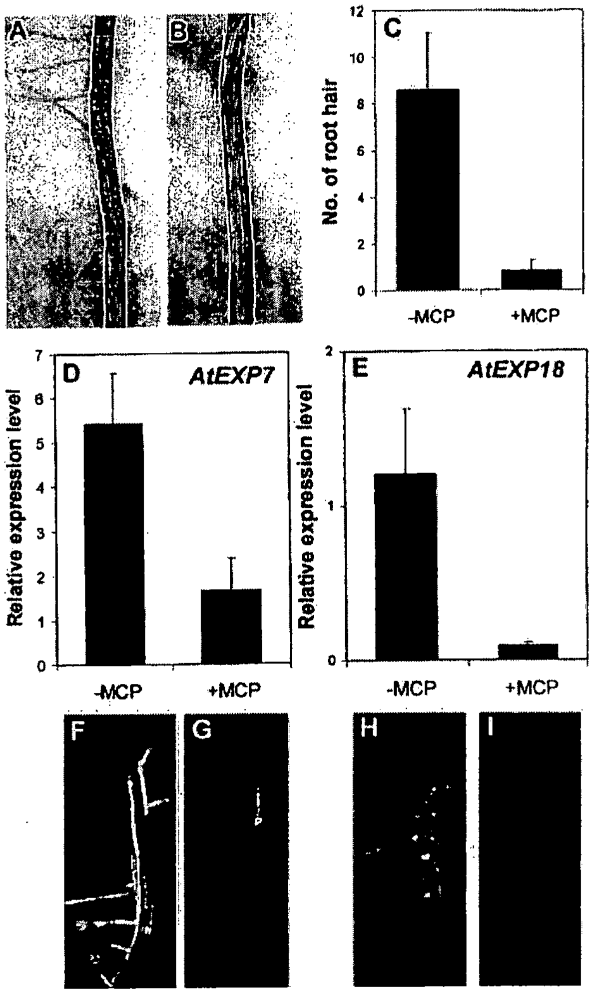

Figure 6. Effect of 1-MCP on Root Separation-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of the roots separated from the agar medium without **(A)** or with **(B)** 1 μL/L 1-MCP.

(C) Effect of 1-MCP on root hair number in separation-treated roots. Total root hairs were counted from the separated region of the root. Bars indicate standard errors (n = 13 to 19).

(D) and **(E)** Relative expression levels of *AtEXP7* **(D)** and *AtEXP18* **(E)** in the root when induced by separation of the root without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 11 to 15).

(F) to **(I)** Confocal microscopy images of roots harboring *AtEXP7* promoter::*GFP* (**[F]** and **[G]**) and *AtEXP18* promoter::*GFP* (**[H]** and **[I]**). Seedlings whose roots were separated from the medium were incubated without (**[F]** and **[H]**) or with (**[G]** and **[I]**) 1-MCP.

previous report (Pitts et al., 1998). Root hair length was decreased significantly in four dominant ethylene receptor mutants (Table 2). Treatment with 1-MCP also greatly decreased root hair elongation in the wild type. Considering the effect of each dominant mutation on root hair length, we can assess the cell type–specific roles of the five ethylene receptors. ETR1 seems to play the most significant role in root hair elongation, followed by ERS1 ≥ ERS2 > ETR2. EIN4 appears to have no function in root hair elongation.

Promoter Analyses of *AtEXP7* and *AtEXP18*

AtEXP7 and *AtEXP18* are expressed specifically in the root hair cell and are induced by ethylene, auxin, and separation of the root from the medium. To define the regulatory elements for the hair cell specificity and effector inducibility of the promoter, we performed promoter analyses of the genes by sequential deletion of the 5′ regions, nucleotide substitution, and gain of function of the *cis* elements. The deleted or substituted promoters were fused directly to the GFP coding sequence, and the gain-of-function *cis* elements were combined with the 35S minimal promoter region of *Cauliflower mosaic virus* (−64 35S promoter; Eyal et al., 1995) that was followed by the GFP sequence. For unambiguous evaluation of the promoter activities, the promoter::*GFP* constructs were introduced stably into plants (wild type and *rhd6*) by Agrobacterium transformation. To assess the inducibility of promoter activities by ethylene, auxin, and root separation, we treated the transformed *rhd6* plants with 5 μM ACC, 30 nM IAA, or separation of the root. Promoter activity was evaluated by confocal laser scanning microscopy to measure GFP fluorescence in roots of the first generation of transformants (9 to 62 independent T1 lines per construct, with an average of 28). The histogram function of Adobe Photoshop was used to quantify the relative GFP fluorescence.

For 5′ deletion analysis of the *AtEXP7* promoter, sequential deletions from −1380 to +48 bp, relative to the predicted transcription initiation site, were generated (Figure 8A). Deletions to −386 bp did not significantly affect the promoter activity in either the wild type or *rhd6* treated with ACC (Figures 8B and 8C). Further deletion to −245 bp decreased promoter activity by 50 to 70% in both backgrounds, and this level continued through additional deletions to −134 bp. In auxin-treated *rhd6* transformants, the promoter activity decreased gradually in deletions from −386 to −134 bp, where ~50% of the activity remained (Figure 8D). Root separation treatment of *rhd6* also gave a similar result, except that the deletion to −386 bp decreased the promoter activity significantly (Figure 8E).

Although the promoter activity was reduced considerably by deletion to −245 bp, both the cell specificity and the inducibility by effectors were maintained until deletion to −134 bp, and no novel expression patterns were observed in other tissues (data not shown). The cell specificity and the

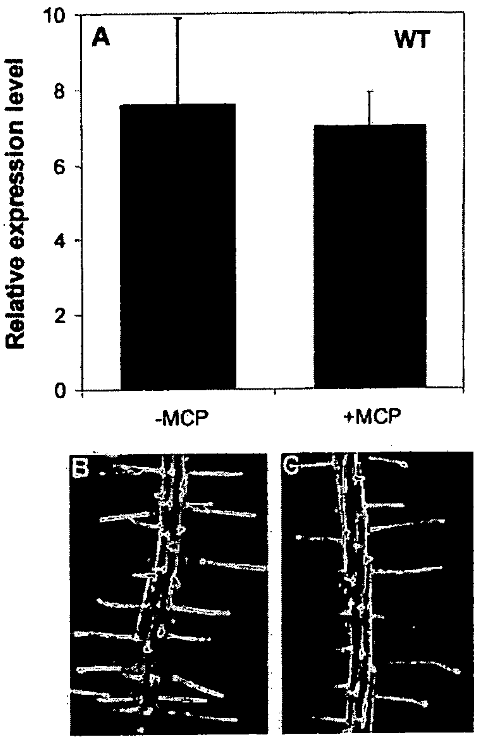

Figure 7. Effect of 1-MCP on Expansin Gene Expression and Root Hair Formation in the Wild Type.

(A) Relative expression levels of *AtEXP7* without (−MCP) or with (+MCP) 1 μL/L 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter. Bars indicate standard errors (*n* = 7 to 10). WT, wild type.

(B) and **(C)** Confocal microscopy images of roots harboring *AtEXP7* promoter::*GFP* without **(B)** or with **(C)** 1 μL/L 1-MCP.

inducibility by effectors disappeared completely with deletion to −70 bp. Although elements for auxin (TGTCTC; −808 bp) and ethylene (AATTCAAA; −615 bp) response are located on the *AtEXP7* promoter, deletions of those elements did not affect the responsiveness of the promoter to these hormones (Figures 8C and 8D). Deletion analysis of the *AtEXP7* promoter suggested that the elements for cell specificity and inducibility by these effectors are located between −134 and −70 bp. In this region (Figure 9A) are three repeats of a core binding sequence (AAAG) for the DOF zinc finger protein (Yanagisawa and Schmidt, 1999) and one core motif (GGATA) for MYBSt1, a MYB-like protein (Baranowskij et al., 1994). The distal region between −386 and −245 bp likely contains some enhancing elements, because deletion of this region reduced promoter activity significantly. A MYBSt1 core motif also is found in this distal promoter region (−281 to −276 bp).

To define the *cis*-regulatory elements in the proximal region (−134 to −70 bp) of the *AtEXP7* promoter, seven 9- to 10-bp-long substitution mutations were introduced into this region. To acquire the greatest mutational effects, an A/T base pair was changed to G/C or C/G. Substitution mutations by ~9 to 10 bp are small enough to localize the controlling elements with reasonable precision (Carey and Smale, 2000). These substitutions replaced the DOF and MYBSt1 core elements and their flanking regions. The substitution mutations E7M1~E7M7 were produced from the −386-bp deletion so that the wild-type promoter had full activity (Figure 9A). Although promoter activity fluctuated between 50 and 130% compared with wild-type (−386 bp) activity, the substitutions E7M1~E7M5 did not greatly diminish promoter activity in either wild-type or ACC-treated *rhd6* roots (Figures 9B and 9C). However, both E7M6 (which includes the MYBSt1 core element) and E7M7 (flanking E7M6) decreased the activity to ~13 to 26%. Similar results were obtained by treatment with auxin or root separation (data not shown). These results suggest that the 19-bp motif containing the MYBSt1 element (hereafter called the −80/−62 element) is most important for both hair cell specificity and inducibility by ethylene, auxin, and root separation.

A gain-of-function analysis was performed to confirm that the identified elements are able, in isolation, to direct hair cell specificity. E7G1~E7G3 are short sequences that contain the proximal MYBSt1 core with different 3′ extensions, and E7G4 includes the entire proximal region between −134 and −46 bp. E7G4M6 and E7G4M7 are the same as E7G4 except that they harbor E7M6 and E7M7 substitution mutations, respectively (Figure 9A). The results shown in Figures 9D to 9G are from the wild-type background, but similar results were obtained with ACC-treated *rhd6* (data not shown). The 35S minimal promoter (mp35S) alone did not show GFP expression (Figure 9E). The gain-of-function promoter constructs E7G1~E7G3 showed promoter activity

Table 2. Root Hair Length in Wild-Type and Ethylene Mutant Plants with Ethylene Precursor or Inhibitor Treatment

Plant	No Treatment	ACC (5 μM)	1-MCP (1 μL/L)
Columbia	0.91 ± 0.22	1.07 ± 0.14	0.51 ± 0.19
etr1-1	0.26 ± 0.14	0.21 ± 0.20	0.27 ± 0.16
etr2	0.72 ± 0.20	0.66 ± 0.08	0.65 ± 0.09
ers1	0.40 ± 0.14	0.41 ± 0.12	0.39 ± 0.13
ers2-1	0.47 ± 0.20	0.47 ± 0.19	0.35 ± 0.11
ein4	0.92 ± 0.12	0.95 ± 0.12	0.56 ± 0.13
ein2-1	0.04 ± 0.02	0.05 ± 0.03	N.D.[a]
etr1-7	0.82 ± 0.07	N.D.	N.D.
ctr1-1	1.22 ± 0.18	N.D.	N.D.

Values shown are means ± SD in mm (*n* = 35).
[a] N.D., not determined.

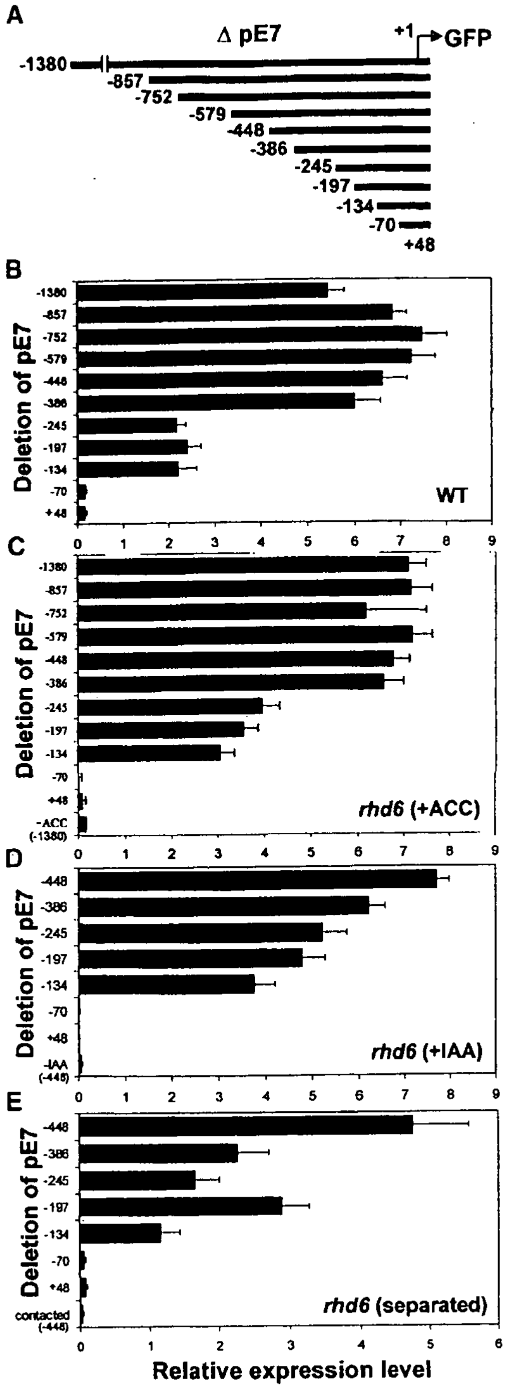

Figure 8. Deletion Analysis of the *AtEXP7* Promoter.

(A) Deletions of the *AtEXP7* promoter (ΔpE7) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP7* promoters in the wild-type (WT) root. Bars indicate standard errors.

(C) to **(E)** Relative activities of the truncated *AtEXP7* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings were treated with 5 μM ACC **(C)** or 30 nM IAA **(D)** or roots were separated from agar to expose them to air **(E)** for 1 day before observation. Bars indicate standard errors.

In **(B)** to **(E)**, n = 27 to 62.

as weak as that of mp35S (Figure 9D), but 20 to 30% of T1 lines from these constructs showed very low and irregular GFP fluorescence in root hair cells (see supplemental data online), which was undetectable in mp35S roots. No significant differences in promoter strength among E7G1~E7G3 were found. By contrast, E7G4 could direct strong hair cell–specific expression of the reporter gene (Figures 9D and 9F). However, the substitution mutation of the E7G4 promoter fragment at the E7M6 or E7M7 site eliminated the promoter activity almost completely (Figures 9D and 9G). This gain-of-function promoter analysis demonstrates that the −80/−62 element confers the hair cell specificity of the *AtEXP7* promoter. However, some additional elements in the proximal region, particularly between −134 and −81 bp, seem to be required for strong promoter activity. These additional elements could be functionally redundant, because the individual substitution mutations (E7M1~E7M5) elsewhere than in the −80/−62 region did not reduce promoter activity substantially (Figures 9B and 9C).

For analysis of the *AtEXP18* promoter, deletions between −1016 and +42 bp were generated (Figure 10A). The *AtEXP18* promoter activity maintained its full strength until the deletion to −241 bp and showed an ~50% decrease by further deletion to −196 bp in both wild-type and ACC- or IAA-treated *rhd6* seedlings (Figures 10B to 10D). The deletions beyond −145 bp completely eliminated the promoter activity in both the wild type and *rhd6* with effector treatments. A similar change of promoter activity was observed in root separation–treated *rhd6*, except that the deletion to −321 bp reduced the activity significantly (Figure 10E). Deletion analysis of the *AtEXP18* promoter indicated that the −196/−145 region contains elements for hair cell specificity and the −241/−196 region may include some enhancing elements relevant to promoter strength. The −196/−145 region of *AtEXP18*, resembling the −80/−62 element of *AtEXP7*, is likely the target of signals from ethylene, auxin, and root separation, because these effectors all require this region for gene induction (Figure 11). However, the −196/−145 region of *AtEXP18* does not contain the MYBSt1 binding motif or similar sequences found in the −80/−62 element of *AtEXP7*. This difference indicates that the cell specificity of the two promoters probably is determined by different transcription factors, which nevertheless are regulated similarly by developmental factors, auxin, ethylene, and root separation.

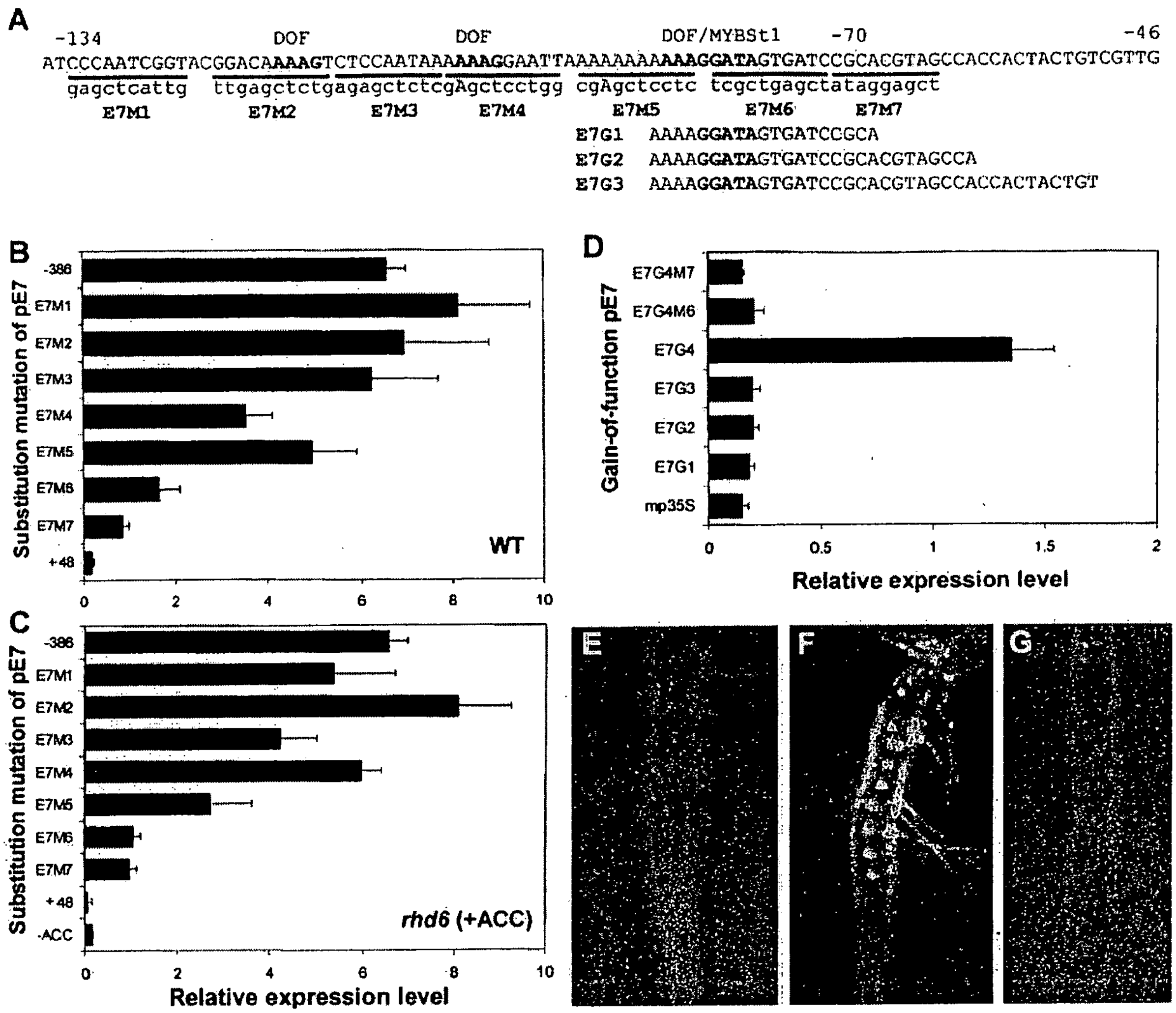

Figure 9. Substitution and Gain-of-Function Analyses of the *AtEXP7* Promoter.

(A) The proximal promoter region of *AtEXP7* between −134 and −46 bp. For substitution mutations (E7M1~E7M7), the underlined regions were replaced by the nucleotides shown in lowercase letters. These substitution mutations were generated from the region between −386 and +48 bp. E7G1~E7G3 are the gain-of-function promoter fragments. The substituted promoters were fused to the coding region of GFP, and the gain-of-function promoter fragments were connected to the minimal 35S promoter of *Cauliflower mosaic virus* (mp35S) before the GFP gene. The putative DOF (AAAG) and MYBSt1 (GGATA) core motifs are indicated.

(B) and **(C)** Relative activities (GFP expression) of the substituted *AtEXP7* promoters in the wild-type (WT) root **(B)** and in the *rhd6* root with 5 μM ACC treatment **(C)**. Bars indicate standard errors (n = 15 to 32).

(D) Relative activities of the gain-of-function *AtEXP7* promoters in the wild-type root. Bars indicate standard errors (n = 9 to 14). E7G4 contains the −134/−46 region (wild-type promoter), and E7G4M6 and E7G4M7 are the same as the E7G4 construct but with E7M6 and E7M7 substitution mutations, respectively.

(E) to **(G)** Confocal microscopy images of roots harboring the gain-of-function *AtEXP7* promoters mp35S **(E)**, E7G4 **(F)**, and E7G4M6 **(G)** (a similar expression pattern was observed with E7G4M7).

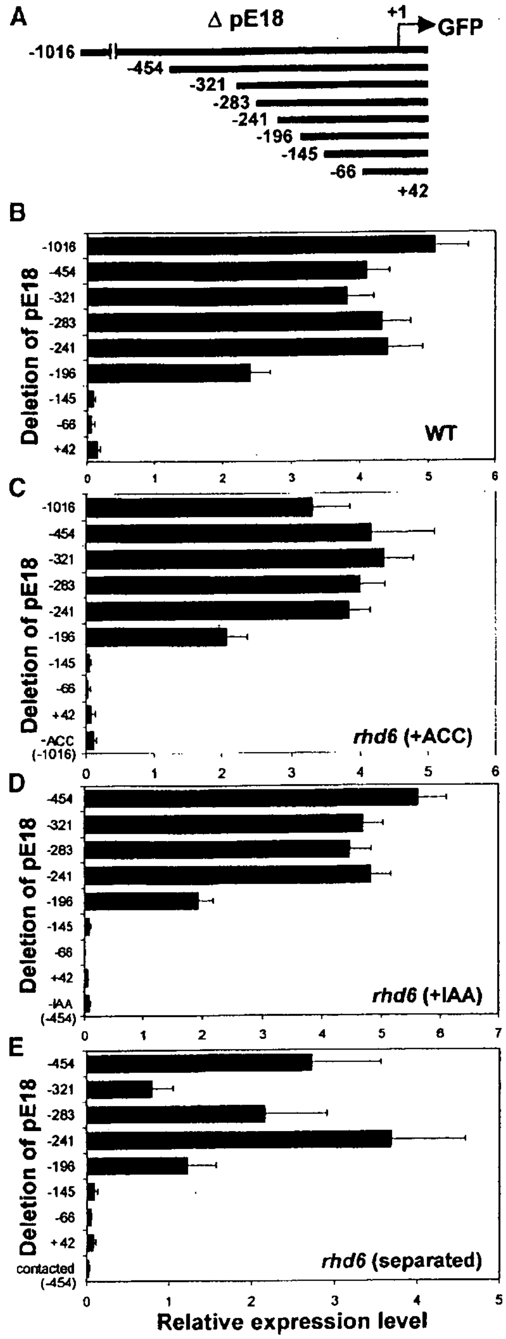

Figure 10. Deletion Analysis of the *AtEXP18* Promoter.

(A) Deletions of the *AtEXP18* promoter (ΔpE18) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP18* promoters in the wild-type (WT) root. Bars indicate standard errors.

(C) to **(E)** Relative activities of the truncated *AtEXP18* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings were treated with 5 μM ACC **(C)** or 30 nM IAA **(D)** or roots were separated from the agar medium **(E)** for 1 day before observation. Bars indicate standard errors.

In **(B)** to **(E)**, n = 25 to 40.

DISCUSSION

Ethylene and Root Hair Development

Recent studies have contributed significantly to our understanding of cell fate determination in the Arabidopsis root epidermis. However, the morphogenetic process of root hair development, which is regulated by hormones and environmental factors, has remained less characterized. In this study, we examined the role of endogenous ethylene and the hierarchical relationship between ethylene, auxin, and an environmental factor (root separation from the agar medium) in root hair initiation. To understand the action of these factors at the gene regulation level, we adopted two expansin genes, *AtEXP7* and *AtEXP18*, whose expression is linked tightly to root hair initiation, as molecular markers.

The involvement of ethylene in root hair formation has been demonstrated in genetic and pharmacological studies. Treatment with the ethylene precursor ACC and mutations of *ctr1* and *eto* induced additional root hairs from the cells in the N position (Dolan et al., 1994; Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995; Cao et al., 1999) (Table 1), and these factors also could restore root hairs in the root hair–defective *rhd6* mutant (Masucci and Schiefelbein, 1996). Although these results clearly show that ethylene is a positive effector of root hair formation, they are indicative of the effect of a constitutive ethylene response and excessive (or exogenous) ethylene but not of the effect of the normal endogenous ethylene level. The mutation of *CTR1* causes constitutive ethylene responses regardless of the absence or presence of ethylene, and the *eto* mutants produce excessive ethylene from 2- to 100-fold (Kieber et al., 1993).

The ethylene biosynthesis inhibitor AVG has been used to show the role of endogenous ethylene, which greatly inhibits root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995) (Table 1). However, AVG likely has toxicity to root hair development, because it completely inhibited root hair formation and the expression of *AtEXP7* and *AtEXP18*, even in the *ctr1* mutant (Figure 3, Table 1).

The role of endogenous ethylene in the wild type can be assessed by the use of mutations that block the ethylene responses. A previous study reported that the dominant ethylene receptor mutant *etr1* maintains normal root hair density (Masucci and Schiefelbein, 1996), thereby raising doubt about the role of endogenous ethylene during the normal (default) process of root hair formation. In Arabidopsis, there are five ethylene receptors whose physiological function, in

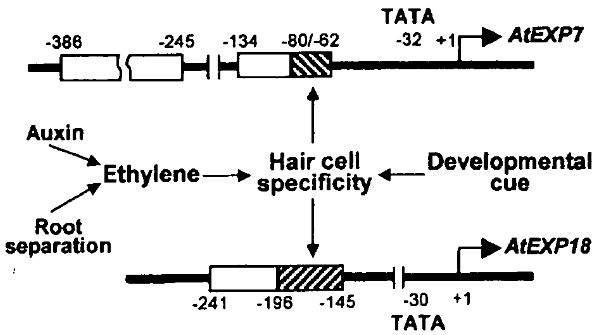

Figure 11. Summary of Promoter Analyses of *AtEXP7* and *AtEXP18*.

The hatched boxes represent elements for hair cell specificity, and the open boxes represent elements that are likely to be relevant to promoter strength. The environmental (root separation) and hormonal signals converge on the elements for hair cell specificity. Numbers indicate nucleotide positions relative to the transcription initiation site (+1). TATA indicates the TATA box.

terms of the triple response, is similar. Dominant mutations in these receptors negatively regulate ethylene responses by constitutively activating CTR1, the negative regulator of downstream ethylene responses. Thus, a dominant mutation in any one of the receptors is able to suppress ethylene responses (Hua and Meyerowitz, 1998). In spite of this genetic principle, inhibition by the dominant mutation shows a dosage-dependent response according to the number of mutant loci and also shows different degrees of phenotypic effect among the five receptors (Hall et al., 1999). Therefore, the contribution of ethylene receptors to root hair formation might depend on receptor species and their temporal/spatial expression pattern.

To determine whether ethylene receptors other than ETR1 are involved in root hair formation, we examined the effect of dominant mutations in all five ethylene receptors. Furthermore, because multiple receptors might be involved in root hair formation, the specific ethylene antagonist 1-MCP was used to simultaneously inhibit ethylene binding by different ethylene receptors. Our results showed that neither the dominant mutations of ethylene receptors nor 1-MCP treatment substantially reduced root hair numbers and the expression of *AtEXP7* and *AtEXP18* (Figures 3 and 7, Table 1), indicating that endogenous ethylene is not required for normal (default) root hair formation in the wild type.

Ethylene, however, is likely to mediate auxin- or root separation–induced root hair formation. Blocking the ethylene perception by 1-MCP almost completely inhibited auxin- or root separation–induced root hair formation and expression of *AtEXP7* and *AtEXP18* (Figures 5 and 6). Auxin and certain biotic/abiotic factors, such as pathogens, wounding, chilling, hypoxia, and water stress, are well-known stimulators of ethylene biosynthesis (McKeon et al., 1995). Localized water stress could develop in the root when it is separated from the agar medium or exposed to air, a treatment that is known to stimulate root hair elongation (Okada and Shimura, 1994). Therefore, auxin and root separation may induce root hair initiation through an increase in ethylene production, although we do not exclude the possibility that these treatments affect components of ethylene signaling.

A previous study suggested that ethylene and auxin take separate pathways to affect root hair development. Auxin restored root hairs in the AVG-treated root and in the *aux1 etr1* double mutant (Masucci and Schiefelbein, 1996). However, the latter case indicates a complicated aspect of root hair development, because ACC significantly suppressed root hair formation in the double mutant rather than simply having no effect on the restoration of root hairs. A similar perplexing result from the same study is that ACC also inhibited root hair formation considerably in the *rhd6 ein2* double mutant. It appears that excessive ethylene (or its precursor) inhibits the ethylene responses of ethylene-insensitive mutants in certain conditions.

The dominant mutant *axr2* maintains 64% of root hairs compared with the wild type, and ACC or auxin only partially restores the root hair number in the mutant (74 to 81% compared with the wild type) (Masucci and Schiefelbein, 1996). The *axr2* mutant carries the gain-of-function mutation in an Aux/IAA transcriptional repressor (IAA7) so that the mutant molecule is resistant to the auxin-mediated degradation process (Nagpal et al., 2000; Tiwari et al., 2001). The *axr2* plant can be less sensitive to ACC if AXR2/IAA7 represses expression of the components of ethylene signaling or if the gain-of-function mutant protein finds new targets, such as genes required for the root hair initiation machinery, as a result of its durability time and concentration in the nucleus. The epistatic effect of *axr2* over *ttg* or *gl2* (Masucci and Schiefelbein, 1996) could be acquired if the latter case occurs.

In contrast to root hair initiation, root hair elongation is dependent on endogenous ethylene. Blocking ethylene perception by gain-of-function mutations of the ethylene receptors or by 1-MCP markedly inhibited root hair elongation (Table 2). The difference in ethylene action on the initiation and elongation of root hairs leads us to propose that the two responses have different sensitivities to ethylene. Root hair initiation may require a higher ethylene level than does the root hair elongation process. Treatment with ACC, auxin, or other stimuli is required to exceed the ethylene concentration needed to stimulate root hair initiation, whereas the lower endogenous ethylene level is sufficient to regulate root hair elongation. Alternatively, it is conceivable that ethylene biosynthesis increases during root hair elongation. Genetic studies indicate that different sets of gene products are instrumental for the root hair initiation and root hair elongation steps (Parker et al., 2000; Schiefelbein, 2000). This finding implies that the two ethylene-dependent responses in a single root hair cell result from the activation of different genetic pathways by different ethylene levels.

Two Different Pathways Manipulate Root Hair Initiation

RHD6 is likely to be a major regulator in the developmental pathway (through TTG/GL2) for root hair formation. Defects in the negative regulator TTG or GL2 induce root hairs from the cells in the N position as well as in the H position. However, root hair numbers in *ttg* and *gl2* mutants are reduced greatly by the defect in RHD6, indicating that RHD6 is an important downstream regulator of the TTG/GL2 pathway (Masucci and Schiefelbein, 1996). The fact that auxin, ethylene, and root separation can restore root hairs in *rhd6* led us to a scheme, illustrated in Figure 12, whereby the separate environmental/hormonal signaling pathway converges with the normal developmental pathway downstream of RHD6. We show the environmental signal (root separation from the medium) as separate from the auxin pathway because root separation restores normal root hair growth in the *aux1* mutant (Okada and Shimura, 1994).

However, the environmental/hormonal pathway appears to have a differential influence on the two epidermal positions (H and N). This is seen clearly in the *rhd6* background (Table 1), in which 5 μM ACC stimulated root hair formation in the H position but had negligible effect in the N position. Even higher levels of ACC (50 μM), as well as the *ctr1* and *eto* mutations, induced only some of the cells in the N position to form root hairs (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999) (Table 1). This differential response could result from a lower ethylene (or ACC) sensitivity of cells in the N position compared with cells in the H position (Dolan, 1997; Cao et al., 1999).

Because the occasional root hairs in *rhd6* emerge in abnormal cell positions, RHD6 was implicated in the control of hair cell polarity (Masucci and Schiefelbein, 1994). Cell specification seems to be normal in the *rhd6* mutant, because the distinctive cytoplasmic characteristics between H- and N-positioned cells are the same as in the wild type; apparently, only the hair-inducing machinery is impaired (Masucci and Schiefelbein, 1996). We found that the *rhd6* mutation inhibited the expression of both *AtEXP7* and *AtEXP18* almost completely (Figures 2K, 3, 8, and 10), suggesting that the molecular function of RHD6 is to regulate gene expression in the root hair cell either as a transcriptional regulator or as its upstream component. RHD6 may regulate the expression of the hair cell genes necessary for hair initiation, such as those involved in cytoskeletal dynamics, localized secretion, wall loosening, and wall synthesis. However, RHD6 probably does not target *AtEXP7* and *AtEXP18* directly, because expansin gene expression in *rhd6* can be restored by hormonal and environmental treatments. Our promoter analyses of the two expansin genes showed that ethylene, auxin, and root separation signals require the same promoter elements that control cell specificity (Figure 11). Thus, we propose that the signals from the developmental and environmental/hormonal pathways are merged at or before the transcription regulators that direct the hair cell specificity of the expansin genes. Identification and characterization of these transcription regulators will be important for understanding the mechanism of pattern formation in the root epidermis.

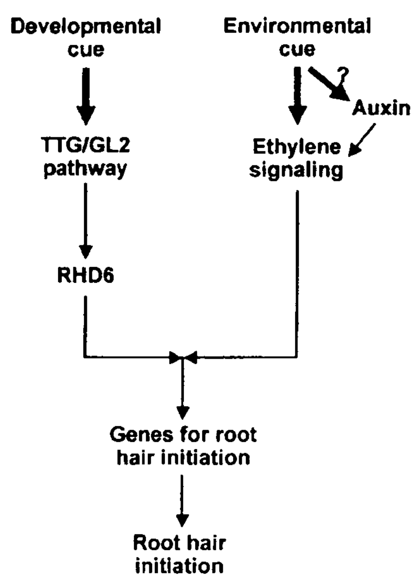

Figure 12. Model Illustrating How Two Separate Signaling Pathways from Developmental and Environmental Cues Merge to Regulate Root Hair Initiation in Arabidopsis.

Arrows designate the information flow.

METHODS

Plant Materials

Arabidopsis thaliana was the model plant in this study. Unless indicated otherwise, the wild type was the Columbia ecotype. The mutant seeds of *ttg-1* (CS89), *gl2-1* (CS65), *eto2* (CS8059), *etr1-1* (CS237), *ers2-1* (CS8854), *ein4* (CS8053), *ctr1-1* (CS8057), and *ein2-1* (CS3071) were obtained from the ABRC (Columbus, OH). *rhd6* seeds were obtained from J.W. Schiefelbein (University of Michigan, Ann Arbor, MI). Seeds of the gain-of-function mutants *etr2* and *ers1* (Hua et al., 1995) were obtained from J. Hua (Cornell University, Ithaca, NY), and seeds of the loss-of-function mutant *etr1-7* were from E. Schaller (University of New Hampshire, Durham, NH). The seeds were sowed on agar plates including 4.3 g/L Murashige and Skoog (1962) nutrient mix (Sigma), 1% Suc, 0.5 g/L Mes, pH 5.7, with KOH, and 0.8% phytagar. After vernalization for 3 days, the seeds were germinated at 23°C under continuous light. For pharmacological experiments, 3-day-old seedlings were transferred to new plates containing growth regulators or antagonists and incubated for 1 additional day, after which root hairs and reporter gene expression patterns were examined. Transformed plants were selected on hygromycin-containing plates (10 μg/mL).

RNA Gel Blot Analyses

Total RNA preparation and RNA gel blot analyses were conducted as described previously (Cho and Kende, 1997). Gene-specific probes for *AtEXP7* and *AtEXP18* were generated from 3′ untranslated regions of the genes. To confirm equal amounts of RNA loading, the membranes were rehybridized with the Arabidopsis actin2 probe. Transcript levels were quantified from autoradiographs using Adobe Photoshop (Adobe Systems, San Jose, CA) as described previously (Cho and Cosgrove, 2000).

Reporter Gene Constructs

For the reporter gene constructs, the *AtEXP7* promoter region (between −1380 and +48 bp relative to the predicted transcription initiation site) from Arabidopsis BAC F5O11 was inserted into HindIII-XbaI sites of the binary vector pGPTV-HYG (Becker et al., 1992), which resulted in the *AtEXP7* promoter::*uidA* (β-glucuronidase [*GUS*]) construct. For the *AtEXP7* promoter::green fluorescent protein (*GFP*) construct, the *uidA* gene was replaced with the gene for GFP. The coding region of GFP was obtained from the pEGFP vector (Clontech, Palo Alto, CA) by PCR using primers 5′-AGTTGGAGCTCTCGAGTCGC-3′ (with the SacI site) and 5′-ATCCCCGGGTACCGGTC-3′ (with the SmaI site). This fragment of the GFP coding region replaced the *uidA* gene between the SacI and SmaI sites of the *AtEXP7* promoter::*GUS* construct. For the *AtEXP7* promoter::*AtEXP7-GFP* construct, in which the *AtEXP7* promoter directs the expression of the AtEXP7-GFP fusion protein, the coding region of *AtEXP7* was amplified from the genomic *AtEXP7* clone by PCR using primers 5′-CCTAAGAATCTAGAAAAAGAGGCTAGAATG-3′ (with the XbaI site) and 5′-AAAAGCCCGGGTAACACGGAAATTAGC-3′ (with the SmaI site). This fragment was inserted into XbaI-SmaI sites of the *AtEXP7* promoter::*GFP* construct. All of the constructs were confirmed by DNA sequencing. The constructs were introduced into Arabidopsis plants by *Agrobacterium tumefaciens* strain C58C1 (pMP90) using the vacuum infiltration method (Bechtold and Pelletier, 1998).

Detection of Reporters

GUS staining was performed as described previously (Cho and Cosgrove, 2000). For the detection of GFP, fluorescence from the seedling root was observed with a confocal laser scanning microscope (LSM-410; Carl Zeiss, Jena, Germany). For the cross-sectional view, 1- to 2-mm root sections were made after embedding the root in 1% agarose. To outline the cell boundary in some samples, the root was stained with propidium iodide (10 μg/mL). Green fluorescence was detected by excitation at 488 nm and emission at 543 nm. Red fluorescence from propidium iodide was detected by excitation at 568 nm and emission at 617 nm. Fluorescence images of the separate channels were digitized with LSM software version 3.5 (New Freedom, PA) and merged using Adobe Photoshop. The false red and green colors were adopted for propidium iodide and GFP fluorescence, respectively.

Observation of Root Hair Number and Length

The number of root hairs was determined using a differential interference contrast microscope according to Masucci and Schiefelbein (1996) with some modifications. For each seedling root, 5 consecutive epidermal cells from the same cell file were observed, and a total of 20 cells from two hair cell files and the adjacent two nonhair cell files were counted. Seven to 13 roots (for a total of 140 to 260 cells) per treatment or genotype were scored. Any protrusion was scored as the presence of the root hair, regardless of the length. In the root separated from the agar medium, total root hairs from the separated region were counted. For root separation, the agar medium immediately below the root tip was cut out, and the root was left to grow to the air. Root hair length was measured using a stereomicroscope when the root hair reached the maximum length. Seven root hairs per plant and five plants per genotype or treatment (for a total of 35 root hairs) were scored.

Treatment of 1-Methylcyclopropene

SmartFresh (0.14% 1-methylcyclopropene [1-MCP]) was obtained from H. Warner at Rohm and Haas (Spring House, PA). 1-MCP gas was produced by mixing the powder with water in a tightly sealed container according to the manufacturer's protocol. The gas was administered to the seedlings so that the final concentration was 1 or 10 μL/L in the container.

Promoter Analyses

The mutated *AtEXP7* promoters with 5′ deletions were prepared by PCR using the same reverse primer (5′-GGACCCATTCTAGACTCTTT-3′, containing the XbaI site) from the 3′ end (+48 bp) and the forward primers (containing the HindIII site) from the various 5′ ends, as indicated in Figure 8A, with the Arabidopsis BAC F5O11 clone as a template. Deletion of the *AtEXP18* promoter was performed similarly by PCR using a reverse primer (5′-TTTACTCTAGATTCTTGAGGGCGCCT-3′, containing the XbaI site) from the 3′ end (+42 bp) and the forward primers (containing the HindIII site) from the 5′ ends, as shown in Figure 10A, with the Arabidopsis BAC F16P17 clone as a template.

Substitution mutagenesis of the proximal region (−134 to −70 bp) of the *AtEXP7* promoter, designated E7M1~E7M7 in Figure 9A, was performed using the "megaprimer PCR" method (Barik, 1995). The megaprimers were amplified using the forward primer 5′-TAGTTAAGCTTTGGAAACGTAA-3′ (located at −386 bp and containing the HindIII site) and the mutagenized reverse primers from the regions indicated in Figure 9A. The second PCR was performed with these megaprimers and the same reverse primer that was used for the deletion analysis.

The gain-of-function promoters of *AtEXP7* were made by associating diverse lengths of proximal promoter parts with the minimal 35S promoter of *Cauliflower mosaic virus* (mp35S). The mp35S region (−64 35S promoter; Eyal et al., 1995) was produced by PCR using the forward primer 5′-AAGGGTCTAGACACAATCCCACTA-3′ (containing the XbaI site) and the reverse primer 5′-GACCACCCGGGGATCCACTA-3′ (containing the SmaI site) from the pBI121 vector (Clontech). This PCR product was inserted between the XbaI and SmaI sites of pGPTV-HYG so that mp35S was followed by the GFP reporter gene. The gain-of-function *AtEXP7* promoters E7G1 to E7G3, as shown in Figure 9A, were prepared by complementing the sense and antisense oligonucleotides, which contained HindIII and XbaI sites at their 5′ and 3′ flanking regions, respectively. The E7G4 gain-of-function promoter was produced by PCR amplification of the region between −134 and −46 bp. E7G4M6 and E7G4M7 were

made from the same region from which E7G4 was made except that PCR was performed with the E7M6 and E7M7 constructs as templates, respectively.

The truncated or substituted promoter fragments were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector so that the promoters were followed by the GFP reporter gene, and the gain-of-function promoters were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector containing mp35S::GFP. The constructs were introduced into Arabidopsis plants (either the wild type or *rhd6*) using Agrobacterium as described above. The first generation of transformants (T1, 9 to 62 independent lines per construct) was used to quantify the relative expression levels of GFP in the root. After selection for 5 days on hygromycin-containing plates, the transformants were transferred to new plates without effectors for the wild-type background or with 1-aminocyclopropane-1-carboxylic acid (5 μM), indole-3-acetic acid (30 nM), or separation of the root from the agar medium for the *rhd6* mutant, and GFP expression was observed 1 day after the treatments.

To evaluate the promoter activity (GFP expression), fluorescence images of roots were taken digitally using a confocal laser scanning microscope. Relative brightness from the digital images was quantified using the histogram function in Adobe Photoshop. For the histogram analysis, a rectangular marquee (4 × 3 of the root diameter) was located around the root, where GFP fluorescence is maximal, and the mean value was read from the histogram window. The final relative brightness was calculated by subtracting the background values.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the genes described in this article are AC025416 (*AtEXP7*), AC011000 (*AtEXP18*), and U41998 (*AtACT2*).

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Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol. Gen. Genet. **222**, 377–383.

Yanagisawa, S., and Schmidt, R.J. (1999). Diversity and similarity among recognition sequences of Dof transcription factors. Plant J. **17**, 209–214.

```
Qy     1981 GAAAACTTCATCTTGTCTGATCTCACACCACATATCCCATTCGCGCTCAACCAACTGATC 2040
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     1981 GAAAACTTCATCTTGTCTGATCTCACACCACATATCCCATTCGCGCTCAACCAACTGATC 2040

Qy     2041 TAGAAGTTTAGATCTTGTTTAAATAACCAGCTTATTTGAACGATGTCTACTTATATAGTA 2100
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2041 TAGAAGTTTAGATCTTGTTTAAATAACCAGCTTATTTGAACGATGTCTACTTATATAGTA 2100

Qy     2101 TGTAGAAACTGTGCAAGCAGTGCACGAGCAACTAGATAGTTAAGAAATAAATACAGATAG 2160
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2101 TGTAGAAACTGTGCAAGCAGTGCACGAGCAACTAGATAGTTAAGAAATAAATACAGATAG 2160

Qy     2161 ATAAGAGATAGATAAGCAACAGATATATGTATTAGGATATAGATAGGCAACAGATATATG 2220
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2161 ATAAGAGATAGATAAGCAACAGATATATGTATTAGGATATAGATAGGCAACAGATATATG 2220

Qy     2221 CACGCTGGCAAGAGATAGATATAGATATAGGCACAGATAGAGAGAAAATAAATAGCAGAT 2280
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2221 CACGCTGGCAAGAGATAGATATAGATATAGGCACAGATAGAGAGAAAATAAATAGCAGAT 2280

Qy     2281 AATATATTAATATAGAGATAGATATATTCTCACCACAATCACTACAGTACAACATTCACG 2340
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2281 AATATATTAATATAGAGATAGATATATTCTCACCACAATCACTACAGTACAACATTCACG 2340

Qy     2341 AGTGACCGCGGATGCAGTCGAGAGGACAACCGTACCACGGCGCCTTGCAGAACACTTTCC 2400
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2341 AGTGACCGCGGATGCAGTCGAGAGGACAACCGTACCACGGCGCCTTGCAGAACACTTTCC 2400

Qy     2401 AAGCCCAGAGCCACTACACCAACCACTCTCGGGCTCTGCTCTATTTATGGAGGAGCAGCC 2460
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2401 AAGCCCAGAGCCACTACACCAACCACTCTCGGGCTCTGCTCTATTTATGGAGGAGCAGCC 2460

Qy     2461 AGCTACAGGCTACAGCCGTGGCGAAAGCACACACGGATCAATCACACTCACTCGCGGCCA 2520
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db     2461 AGCTACAGGCTACAGCCGTGGCGAAAGCACACACGGATCAATCACACTCACTCGCGGCCA 2520

Qy     2521 TTGTCCTGCTCGTGCGTGCTCTGCCTT 2547
            |||||||||||||||||||||||||||
Db     2521 TTGTCCTGCTCGTGCGTGCTCTGCCTT 2547
```

```
RESULT 2
AAD25704
ID   AAD25704 standard; DNA; 1505 BP.
XX
AC   AAD25704;
XX
DT   12-MAR-2002  (first entry)
XX
DE   Maize glutamine synthetase (GS1-2) gene.
XX
KW   Maize; transcription regulatory region; nitrogen metabolism; invertase;
KW   glutamine synthetase; seed assimilation; seed development; GS1-2 gene;
KW   amino acid transaminase; fungal resistance; disease resistance;
KW   carbon metabolism; ds.
XX
OS   Zea mays.
XX
FH   Key             Location/Qualifiers
FT   misc_feature    1
FT                   /*tag= a
FT                   /note= "5' deletion start site"
FT   misc_feature    259
FT                   /*tag= b
FT                   /note= "5' deletion start site"
FT   protein_bind    430. .439
FT                   /*tag= c
FT                   /bound_moiety= "c-myb transcription factor"
FT   protein_bind    443. .448
FT                   /*tag= d
FT                   /bound_moiety= "NIT2 transcription factor"
FT   misc_feature    459
FT                   /*tag= e
FT                   /note= "5' deletion start site"
FT   protein_bind    483. .488
FT                   /*tag= f
FT                   /bound_moiety= "NIT2 transcription factor"
FT   CAAT_signal     500. .503
FT                   /*tag= g
FT   misc_feature    593
FT                   /*tag= h
FT                   /note= "5' deletion start site"
FT   misc_feature    603. .608
FT                   /*tag= i
FT                   /note= "P site"
FT   TATA_signal     626. .631
FT                   /*tag= j
FT   misc_feature    631
FT                   /*tag= k
FT                   /note= "5' deletion start site"
FT   5'UTR           665. .767
FT                   /*tag= m
FT   misc_signal     665
FT                   /*tag= l
FT                   /note= "Transcription start site"
FT   exon            768. .840
FT                   /*tag= n
FT                   /number= 1
FT   intron          841. .1222
FT                   /*tag= o
FT                   /number= 1
FT                   /cons_splice= (5'site:NO, 3'site:YES)
FT   exon            1223. .1262
FT                   /*tag= p
FT                   /number= 2
FT   intron          1263. .1469
FT                   /*tag= q
FT                   /number= 2
FT   exon            1470. .1505
FT                   /*tag= r
FT                   /number= 3
FT                   /partial
XX
PN   WO200192465-A2.
XX
PD   06-DEC-2001.
XX
PF   23-MAY-2001; 2001WO-US010830.
XX
PR   25-MAY-2000; 2000US-0206984P.
XX
PA   (BIOT-) BIOTECHNOLOGY RES & DEV CORP.
XX
PI   Muhitch MJ;
XX
DR   WPI; 2002-090042/12.
XX
PT   New maize glutamine synthase gene promoter, useful for affecting seed
PT   assimilation and development (e.g. increasing seed yield and quality) in
PT   plants, and for producing plants with increased fungal and disease
PT   resistance.
XX
PS   Example; Fig 1; 41pp; English.
XX
CC   The invention relates to transcription regulatory region (promoter) for
CC   the maize glutamine synthetase (GS1-2) gene. The GS1-2 gene comprising
CC   the transcription regulatory region is useful for affecting seed
CC   assimilation and development, and for increasing fungal and disease
CC   resistance. It is useful for producing plants with reduced mycotoxin
CC   levels and increased disease resistance, and for increasing seed yield
CC   and quality in plants. It is also useful for encoding proteins involved
CC   in seed development and metabolism, particularly enzymes involved in
CC   carbon and/or nitrogen metabolism (e.g. glutamine synthetases or their
CC   isozymes, amino acid transaminases and invertases). The present sequence
CC   is maize GS1-2 gene
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PG Pub: 20040148651

how do I get this? what IS application #?

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XX
SQ   Sequence 1505 BP; 378 A; 394 C; 286 G; 447 T; 0 U; 0 Other;

  Query Match             10.0%;  Score 254.2;  DB 6;  Length 1505;
  Best Local Similarity   93.6%;  Pred. No. 2.2e-45;
  Matches  265;  Conservative    0;  Mismatches   18;  Indels    0;  Gaps    0;

Qy       2265 AAAATAAATAGCAGATAATATATTAATATAGAGATAGATATATTCTCACCACAATCACTA 2324
              | ||   ||||  |   | |||   |||||||||||||||||||||||||||||||||||
Db        449 ATAAAGTATAGAGGCACAGATAGAGATATAGAGATAGATATATTCTCACCACAATCACTA 508

Qy       2325 CAGTACAACATTCACGAGTGACCGCGGATGCAGTCGAGGACAACCGTACCACGGCGCC 2384
              |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Db        509 CAGTACAACATTCACGAGTGACCGCGGATGCACTCGAGGACAACCGTACCACGGCGCC 568

Qy       2385 TTGCAGAACACTTTCCAAGCCCAGAGCCACTACACCAACCACTCTCGGGCTCTGCTCTAT 2444
              ||||||||||||| ||||||||| ||||| ||||||||||||||||||||||||||||||
Db        569 TTGCAGAACACTTCCCAAGCCCAAAGCCATTACACCAACCACTCTCGGGCTCTGCTCTAT 628

Qy       2445 TTATGGAGGAGCAGCCAGCTACAGGCTACAGCCGTGGCGAAAGCACACACGGATCAATCA 2504
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db        629 TTATGGAGGAGCAGCCAGCTACAGGCTACAGCCGTGGCGAAAGCACACACGGATCAATCA 688

Qy       2505 CACTCACTCGCGGCCATTGTCCTGCTCGTGCGTGCTCTGCCTT 2547
              ||||||||||||||||||||||||| |||||||||||||||||
Db        689 CACTCACTCGCGGCCATTGTCCTGCCCGTGCGTGCTCTGCCTT 731

RESULT 3
AAD25728
ID   AAD25728 standard; DNA; 664 BP.
XX
AC   AAD25728;
XX
DT   12-MAR-2002  (first entry)
XX
DE   Maize glutamine synthetase (GS1-2) gene transcription regulatory region.
XX
KW   Maize; transcription regulatory region; nitrogen metabolism; invertase;
KW   glutamine synthetase; seed assimilation; seed development; GS1-2 gene;
KW   amino acid transaminase; fungal resistance; disease resistance;
KW   carbon metabolism; ds.
XX
OS   Zea mays.
XX
FH   Key             Location/Qualifiers
FT   protein_bind    430..439
FT                   /*tag= a
FT                   /bound_moiety= "c-myb transcription factor"
FT   protein_bind    443..448
FT                   /*tag= b
FT                   /bound_moiety= "NIT2 transcription factor"
FT   protein_bind    483..488
FT                   /*tag= c
FT                   /bound_moiety= "NIT2 transcription factor"
FT   CAAT_signal     500..503
FT                   /*tag= d
FT   misc_feature    603..608
FT                   /*tag= e
FT                   /note= "P site"
FT   TATA_signal     626..631
FT                   /*tag= f
XX
PN   WO200192465-A2.
XX
PD   06-DEC-2001.
XX
PF   23-MAY-2001; 2001WO-US010830.
XX
PR   25-MAY-2000; 2000US-0206984P.
XX
PA   (BIOT-) BIOTECHNOLOGY RES & DEV CORP.
XX
PI   Muhitch MJ;
XX
DR   WPI; 2002-090042/12.
XX
PT   New maize glutamine synthase gene promoter, useful for affecting seed
PT   assimilation and development (e.g. increasing seed yield and quality) in
PT   plants, and for producing plants with increased fungal and disease
PT   resistance.
XX
PS   Claim 1; Fig 2; 41pp; English.
XX
CC   The invention relates to transcription regulatory region (promoter) for
CC   the maize glutamine synthetase (GS1-2) gene. The GS1-2 gene comprising
CC   the transcription regulatory region is useful for affecting seed
CC   assimilation and development, and for increasing fungal and disease
CC   resistance. It is useful for producing plants with reduced mycotoxin
CC   levels and increased disease resistance, and for increasing seed yield
CC   and quality in plants. It is also useful for encoding proteins involved
CC   in seed development and metabolism, particularly enzymes involved in
CC   carbon and/or nitrogen metabolism (e.g. glutamine synthetases or their
CC   isozymes, amino acid transaminases and invertases). The present sequence
CC   is maize GS1-2 gene transcription regulatory region
XX
SQ   Sequence 664 BP; 207 A; 142 C; 112 G; 203 T; 0 U; 0 Other;

  Query Match              7.4%;  Score 188.8;  DB 6;  Length 664;
  Best Local Similarity   92.1%;  Pred. No. 3.3e-31;
  Matches  199;  Conservative    0;  Mismatches   17;  Indels    0;  Gaps    0;

Qy       2265 AAAATAAATAGCAGATAATATATTAATATAGAGATAGATATATTCTCACCACAATCACTA 2324
              | ||   ||||  |   | |||   |||||||||||||||||||||||||||||||||||
Db        449 ATAAAGTATAGAGGCACAGATAGAGATATAGAGATAGATATATTCTCACCACAATCACTA 508

Qy       2325 CAGTACAACATTCACGAGTGACCGCGGATGCAGTCGAGGACAACCGTACCACGGCGCC 2384
              |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Db        509 CAGTACAACATTCACGAGTGACCGCGGATGCACTCGAGGACAACCGTACCACGGCGCC 568

Qy       2385 TTGCAGAACACTTTCCAAGCCCAGAGCCACTACACCAACCACTCTCGGGCTCTGCTCTAT 2444
              ||||||||||||| ||||||||| ||||| ||||||||||||||||||||||||||||||
Db        569 TTGCAGAACACTTCCCAAGCCCAAAGCCATTACACCAACCACTCTCGGGCTCTGCTCTAT 628

Qy       2445 TTATGGAGGAGCAGCCAGCTACAGGCTACAGCCGTG 2480
              ||||||||||||||||||||||||||||||||||||
Db        629 TTATGGAGGAGCAGCCAGCTACAGGCTACAGCCGTG 664

RESULT 4
ADE25029
ID   ADE25029 standard; cDNA; 3001 BP.
XX
AC   ADE25029;
XX
DT   29-JAN-2004  (first entry)
XX
DE   Plant growth associated gene cDNA seq id 4.
XX
KW   plant growth; plant growth trait modulation; Brassicaceae; Arabidopsis;
KW   Brassica; Zea; Oryza; Triticum; Hordeum; Lolium; Sorghum; Glycine;
KW   Medicago; Helianthus; Lactuca; Beta; Vitis; Solanum; Lycopersicon;
KW   Capsicum; Gossypium; Hevea; Linum; Prunus; Citrus; Populus; Pinus;
KW   Quercus; gene; ss.
XX
OS   Magnoliophyta.
XX
PN   US2003188343-A1.
XX
PD   02-OCT-2003.
XX
PF   07-JAN-2003; 2003US-00338777.
XX
PR   09-JAN-2002; 2002US-0347288P.
XX
PA   (LYNX-) LYNX THERAPEUTICS INC.
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X. RELATED PROCEEDINGS APPENDIX

Decision in Appeal No. 2003-0936 (U.S. Ser. No. 09/532,806, now U.S. Patent No. 6,747,189) mailed August 29, 2003

Order in Appeal No. 2005-0409 (U.S. Ser. No. 09/757,089, now allowed) mailed April 29, 2005

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

Paper No. 21

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte DAVID McELROY,
EMIL OROZCO, and LUCILLE LACCETTI

Appeal No. 2003-0936
Application No. 09/532,806[1]

MAILED
AUG 29 2003
U.S. PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS
AND INTERFERENCES

ON BRIEF

Before WINTERS, GRON, and GREEN, Administrative Patent Judges.

GRON, Administrative Patent Judge.

DECISION ON APPEAL UNDER 35 U.S.C. § 134

Introduction

This is an appeal under 35 U.S.C. § 134 of an examiner's final rejections of Claims 1, 4-54, and 85-131, all claims pending in Application 09/532,806, under 35 U.S.C. § 112, first paragraph. Claims 1, 4-54, and 85-113 stand finally rejected for noncompliance with the written description requirement of 35 U.S.C. § 112, first

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[1] Application for patent filed March 21, 2000.

paragraph. Claims 1, 4-54, and 85-131 stand finally rejected for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph (Brief on Appeal, p. 3 (AB 3); Examiner's Answer, p. 3 (EA 3)). Claims 1, 4-20, 33, 34, 45, 46, 48, 49, 51, 52, 85, 96, and 114, reproduced below, are representative of the full scope of the subject matter claimed.

1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

4. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 110 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

5. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 125 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

6. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 250 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

7. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 400 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

8. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 750 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

9. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1000 to about

3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

10. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

11. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

12. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

13. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 3000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

14. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises the nucleic acid sequence of SEQ ID NO:1.

15. The isolated nucleic acid of claim 1, further comprising an enhancer.

16. The isolated nucleic acid of claim 15, wherein said enhancer comprises an intron.

17. The isolated nucleic acid of claim 15, wherein said intron is selected from the group consisting of the rice actin 1 intron and the rice actin 2 intron.

18. The isolated nucleic acid of claim 1, further comprising a terminator.

19. The isolated nucleic acid of claim 18, wherein said terminator comprises an rbcS terminator.

20. A transgenic plant stably transformed with a selected DNA comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

33. The transgenic plant of claim 20, wherein said selected DNA further comprises a selected heterologous coding region operably linked to said GRP promoter.

34. The transgenic plant of claim 33, wherein said selected heterologous coding region encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, male sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

45. The transgenic plant of claim 20, further defined as a monocotyledonous plant.

46. The transgenic plant of claim 45, wherein said monocotyledonous plant is selected from the group consisting of wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane.

48. The transgenic plant of claim 20, further defined as a dicotyledonous plant.

49. The transgenic plant of claim 48, wherein said dicotyledonous plant is selected from the group consisting of tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton.[2]

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[2] We note here that the Markush grouping of dicotyledonous plants twice recites cotton. Counsel should edit all of appellants' claims for typographical errors.

51. The transgenic plant of claim 20, further defined as a fertile R_0 transgenic plant.

52. A seed of the fertile R_0 transgenic plant of claim 51, wherein said seed comprises said selected DNA.

85. A transgenic plant[3] stably transformed with a selected DNA comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

96. The transgenic plant cell of claim 85, wherein said selected DNA further comprises a selected coding region operably linked to said maize GRP promoter.

114. A method of preparing a transgenic plant comprising the steps of:

(i) obtaining a construct comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1;

(ii) transforming a recipient plant cell with said construct; and

(iii) regenerating said recipient plant cell to obtain a transgenic plant transformed with said construct.

According to the examiner, (1) appellants' claims are drawn to "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention" (EA 3); and

---

[3] Note that claims dependent upon Claim 85 refer to "[t]he transgenic plant <u>cell</u> of claim 85" (emphasis added).

(2) "the specification, while being enabling for the isolated nucleic acid that is the maize GRP promoter described in Example 1, transgenic plants and cells comprising said promoter, and method of preparing said transgenic plant, does not reasonably provide enablement for other isolated nucleic acids that are a maize GRP promoter, or transgenic plant, seeds and cells comprising other isolated nucleic acids" (EA 3). In support of the final rejections, the examiner cites Kim, Y., et al., "A 20 nucleotide upstream element is essential for the nopaline synthase (nos) promoter activity," Plant Molecular Biology, Vol. 24, pp. 105-117 (1994), and Benfey, P., et al., "The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants," Science," Vol. 250, pp. 959-966 (1990).

Discussion

1. Rejection for inadequate written description

Figure 4 describes SEQ ID NO:1 by naming its 3536 contiguous nucleotides. In so doing, appellants' specification, as originally filed, prima facie described each and every isolated 95, 110, 125, 250, 400, 750, 1000, 1500, 2000, 2500, and 3000 contiguous nucleotide segment of the 3536 contiguous nucleotides of SEQ ID NO:1 comprising a functional maize GRP promoter. Nevertheless, the examiner is concerned that appellants' specification does not

identify which of all the possible at least 95 contiguous nucleotide segments of the 3536 contiguous nucleotides of SEQ ID NO:1 described are required to promote expression of the various coding regions to be linked thereto. More specifically, the examiner argues (EA 4-5):

> 35 U.S.C. § 112 requires that Appellants describe in some manner the structure of the nucleic acid sequences that would have promoter function. Showing how or why the claimed promoter sequences function, in particular showing which structural features are necessary for the function of the maize GRP promoter, is one way in which Appellants may describe the structure of subfragments of SEQ ID NO:1 that would have promoter function. Appellants might also describe the structure of subfragments of SEQ ID NO:1 that would have promoter function by describing a representative number of species of subfragments having promoter function, so that one skilled in the art would have a basis for recognizing the characteristics of SEQ ID NO:1 subfragments that retain promoter function. Here Appellants have done neither. Appellants describe only a single element that has promoter function. However, this element has only been shown to have promoter activity when linked to the rice act 2 intron 1 deletion derivative. This element appears to be a 639 base pair subfragment of the 3536 base pair sequence of SEQ ID NO:1 (examples 2 and 3 pages 112-114 of the specification, and figures 1 and 2), though it is unclear exactly which nucleotides of SEQ ID NO:1 provided this promoter function, as the specification describes the construction of the promoter containing construct only in terms of the restriction enzymes used to subclone the subfragment of SEQ ID NO:1 into the reporter construct. . . . If a nucleotide required for promoter function is not present in a given sequence, that sequence will no longer exhibit promoter function.

Unlike the specification which describes every subfragment of SEQ ID NO:1 that is between 95 and 3536 contiguous bases long, the

examiner argues that "Claim 1 encompasses every subfragment of SEQ ID NO:1 that is between 95 and 3536 contiguous bases long and that <u>has promoter function</u>" (EA 5; emphasis added). According to the examiner, "the description must allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function" (EA 6).

As we understand the rejection, the examiner concedes that appellants' specification describes every subfragment claimed which can function as a promoter. However, that description does not satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, because the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that can function as a promoter are not distinguished from the subfragments of SEQ ID NO:1 between 95 and 3536 contiguous bases in length that cannot function as a promoter. The problem with the examiner's position is that it confuses the written description requirement of 35 U.S.C. § 112, first paragraph, with the enablement requirement of 35 U.S.C. § 112, first paragraph. For example, in support of the written description requirement of 35 U.S.C. § 112, first paragraph, the examiner states (EA 5-6)(emphasis added):

> While it is not required that Appellant describe exactly the subject matter claimed, the description must allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function. The Examiner maintains that the instant disclosure does not allow those skilled in the art to recognize what regions of SEQ ID NO:1 would need to be retained in its subfragments such that the subfragments could reasonably be expected to retain promoter function . . . .

The examiner's criticism that the specification would not have allowed persons skilled in the art to recognize what nucleotide subfragments of SEQ ID NO:1 function as promoters is indistinct from the concomitant criticism that the specification would not have allowed persons skilled in the art to make and use the nucleotide subfragments of SEQ ID NO:1 as promoters without undue experimentation. However, Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991), instructs at 1562, 19 USPQ2d at 1117:

> This court in [In re]Wilder[, 736 F.2d 1516, 1520, 222 USPQ 369, 372 (Fed. Cir. 1994), cert. denied, 469 U.S. 1209 (1985),] (and the CCPA before it) clearly recognized, and we hereby reaffirm, that 35 USC 112, first paragraph, requires a "written description of the invention" which is separate and distinct from the enablement requirement. The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention.

> The invention is, for purposes of the "written description" inquiry, whatever is now claimed.

More recently, the Federal Circuit discussed both the Vas-Cath and Eli Lilly decisions (Vas-Cath, Inc. v. Mahurkar, supra; and Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997)), in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). The court stated in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1324, 63 USPQ2d at 1613:

> In Eli Lilly, we concluded that a claim to a microorganism containing human insulin cDNA was not adequately described by a statement that the invention included human insulin cDNA. Id. at 1557, 43 USPQ2d at 1405. The recitation of the term human insulin cDNA conveyed no distinguishing information about the identity of the claimed DNA sequence, such as its relevant structural or physical characteristics. Id. We stated that an adequate written description of genetic material "'requires a precise definition, such as by structure, formula, chemical name, or physical properties,' not a mere wish or plan for obtaining the claimed chemical invention," and that none of those descriptions appeared in that patent. Id. at 1566, 43 USPQ2d at 1404 . . . . The specification in the Eli Lilly case did not show that the inventors had possession of human insulin cDNA.

However, the court in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1329, 63 USPQ2d at 1616-17, clarified:

> It is true that in Vas-Cath, we stated: "The purpose of the 'written description' requirement is broader than to merely explain how to 'make and use'; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she

> was in possession of the invention." Vas-Cath, 935 F.2d at 1563-64, 19 USPQ2d at 1117. That portion of the opinion in Vas-Cath, however, merely states a purpose of the written description requirement, viz., to ensure that the applicant had possession of the invention as of the desired filing date. It does not state that possession alone is always sufficient to meet that requirement. Furthermore, in Lockwood v. American Airlines, Inc., we rejected Lockwood's argument that "all that is necessary to satisfy the description requirement is to show that one is "in possession" of the invention. 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Rather, we clarified that the written description requirement is satisfied by the patentee's disclosure of "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention." Id.

Accordingly, we espouse the view expressed in Evans v. Eaton, 20 U.S. (7 Wheat.) 356 (1822), as did the court in Vas-Cath, Inc. v. Mahurkar, 935 F.2d at 1561, 19 USPQ2d at 1114-15, that:

> [T]he Court concluded that the specification of a patent had two objects, the first of which was "to enable artizans to make and use [the invention]. . . ." [Evans v. Eaton,] . . . at 433. The second object of the specification was
>
> > to put the public in possession of what the party claims as his own invention, so as to ascertain if he claims anything that is in common use, or is already known, and to guard against prejudice or injury from the use of an invention which the party may otherwise innocently suppose not to be patented. It is, therefore, for the purpose of warning an innocent purchaser, or other person using . . . [the invention], of his infringement of the patent; and at the same time, of taking from the inventor the means of practicing upon the credulity or the fears of other persons, by

> pretending that his invention is more than what it really is, or different from its ostensible objects, that the patentee is required to distinguish his invention in his specification.

Id. at 434.

In that light, we find that here the isolated nucleic acid maize GRP promoter appellants claim comprising at least 95, 110, 125, 250, 400, 750, 1000, 1500, 2000, 2500, 3000, and 3536 contiguous nucleotide fragments of the 3536 contiguous nucleotides of SEQ ID NO:1 of Figure 4 is so precisely defined in terms of structure, formula, chemical name, and function, including Figures 1-4 and examples, that persons skilled in the art immediately would have understood what appellants claim as their invention and could readily distinguish what appellants claim from anything that is in common use, all that is known, and anything proposed for production and use in the art. If an isolated nucleotide sequence comprises at least 95 contiguous nucleotides of the 3536 contiguous nucleotides of SEQ ID NO:1 and functions as a GRP promoter when operably linked to a coding region, appellants claim it.[4] Whether the inventors "had possession of the claimed

---

[4] The examiner has not adequately explained why appellant's Claims 14, 32, and 95 stand rejected under 35 U.S.C. § 112, first paragraph, for inadequate written description. As per those claims, the GRP promoter comprises the complete nucleic acid sequence of SEQ ID NO:1.

invention" (EA 3), the test the examiner used to determine compliance with the written description requirement of the first paragraph of Section 112, is not the gauge for compliance. As the court instructs in Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d. at 1330, 63 USPQ2d at 1617:

> A showing of "possession" is ancillary to the statutory mandate that "[t]he specification shall contain a written description of the invention," and that requirement is not met if, despite a showing of possession, the specification does not adequately describe the claimed invention.

Accordingly, the examiner erred in rejecting Claims 1, 4-54, and 85-113 for noncompliance with the written description requirement of 35 U.S.C. § 112, first paragraph.

2. Rejection for nonenablement

The examiner's rejection of Claims 1, 4-54, and 85-131, all claims pending in this application, for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, is an entirely distinct issue. At the onset, appellants' own specification teaches that: (1) the art is unpredictable, and (2) a considerable amount of experimentation may be required to enable persons skilled in the art to make and use the full scope of the subject matter claimed; yet the kind and amount of experimentation required to enable one skilled in the art to make

and use the full scope of the subject matter claimed is well within the knowledge and skill of a person with the ordinary level of knowledge and skill in this art to perform without undue experimentation. For example, appellants' specification teaches (Spec., pp. 12-13; emphasis added)):

> [T]he current invention includes sequences which have been derived from the maize GRP promoter disclosed herein. One efficient means for preparing such derivatives comprises introducing mutations into the sequences of the invention, for example, the sequence given in SEQ ID NO:1. <u>Such mutants may potentially have enhanced or altered function relative to the native sequence or alternatively, may be silent with regard to function</u>.
>
> Mutagenesis may be carried out at random and the <u>mutagenized sequences screened for function in a trial-by-error procedure</u>. Alternatively, <u>particular sequences which provide the ZMGRP promoter with desirable expression characteristics could be identified and these or similar sequences introduced into other related or non-related sequences</u> via mutation. <u>Similarly, non-essential elements may be deleted without significantly altering the function of the elements</u>. It further is contemplated that one could mutagenize these sequences in order to enhance their utility in expressing transgenes in a particular species, for example, maize.
>
> The <u>means for mutagenizing a DNA segment encoding a ZMGRP promoter sequence of the current invention are well-known to those of skill in the art</u>. Mutagenesis may be performed in accordance with any of the <u>techniques known in the art</u>, such as, but not limited to, synthesizing an oligonucleotide having one or more mutations within the sequence of a particular regulatory region. In particular, <u>site-specific mutagenesis is a technique useful in the preparation of promoter mutants</u>, through specific mutagenesis of the underlying DNA. <u>The technique further</u>

> provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. . . .

The specification teaches that "the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications" (Spec., p. 14). "The preparation of sequence variants of the selected promoter DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained" (Spec., p. 15)(emphasis added). According to the specification (Spec., pp. 15-16)(emphasis added):

> Examples of such methodologies are provided by U.S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety. A number of template dependent processes are available to amplify the target sequences of interest present in a sample, such methods being well known in the art and specifically disclosed herein below.
>
> One efficient, targeted means for preparing mutagenized promoters or enhancers relies upon the identification of putative regulatory elements within the target sequence. This can be initiated by comparison with, for example, promoter sequences known to be expressed in a similar manner. Sequences which are shared among elements with similar functions or expression patterns are likely candidates for the binding of transcription factors and are thus likely elements which confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by function analysis of each deletion construct

> by assay of a reporter gene which is functionally attached to each construct. As such, once a starting promoter or intron sequence is provided, any of a number of different functional deletion mutants of the starting sequence could be readily prepared.
>
> As indicated above, deletion mutants of the ZMGRP promoter also could be randomly prepared and then assayed. With this strategy, a series of constructs are prepared, each containing a different portion of the clone (a subclone), and these constructs are then screened for activity. A suitable means for screening for activity is to attach a deleted promoter construct to a selectable or screenable marker, and to isolate only those cells expressing the marker protein. In this way, a number of different, deleted promoter constructs are identified which still retain the desired, or even enhanced, activity. The smallest segment which is required for activity is thereby identified through comparison of the selected constructs. This segment may then be used for the construction of vectors for the expression of exogenous protein.

The specification also generally describes various regulatory elements (Spec., pp. 18-21), terminators (Spec., p. 21), transit or signal peptides (Spec., pp. 21-23), marker genes (Spec., pp. 23-27), and exogenous genes for herbicide resistance, insect resistance, environment or stress resistance, disease resistance, mycotoxin reduction, grain quality, etc. (Spec., pp. 27-61), which are suitable for use in modifying plant characteristics, and include citations to prior art and summaries of the state of the art. The specification thereafter discusses assays which may be employed to determine levels of expression of new transgenic DNA

constructs (Spec., pp. 61-108) and various art recognized methods suitable for plant transformation, growth, stabilization, regeneration, seed production, and breeding (Spec., pp. 69-74), including liberal citation of the prior art and discussion of the state of the art. Following the aforementioned teachings, the specification introduces the examples presented as follows (Spec., pp. 110-111)(emphasis added):

> The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in <u>the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention</u>, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. . . .
>
> . . . . .
>
> The current inventors have demonstrated the utility of a novel maize promoter, designated <u>Zea Mays</u> Glycine Rich Protein (ZMGRP) promoter, in conjunction with an intron in transgenic maize. The ZMGRP promoter comes from a gene (Genbank Acc# GI/22312) that is induced in response to water stress and wounding. The ZMGRP mRNA has been shown to accumulate in epidermal cells upon induction (Gomez <u>et al.</u>, 1988). The ZMGRP promoter was isolated from a maize B73 genomic library and fused to the <u>gus</u> reporter gene, both with and without a modified intron from the rice <u>Act2</u> gene (see Example 5).

> Transient expression assays in microparticle bombarded maize suspension cells and in excised maize root and leaf tissue were carried out in order to determine the activity of the ZMGRP promoter. The promoter was shown to be functionally active in conjunction with a modified actin 2 (Act2) intron 1. Furthermore, the ZMGRP promoter - Act2 intron combination yielded transient expression levels that were at least 70% the level observed from the rice actin 1 (Act1) promoter - intron combination (Zhang, W., McElroy, D., Wu, R., 1991). Finally, the ZMGRP promoter - intron - gus construct was shown to express high levels of GUS protein in the leaves, stems and meristematic regions of the roots of R_0 maize plants regenerated from transformed maize callus.

Accordingly, appellants argue that the broad teachings of the specification and claims are supported by a number of specific examples of isolated DNA comprising at least 95 contiguous bases of SEQ ID NO:1 which comprise a functional maize GRP promoter. We examine those examples below.

Example 1 teaches that the inventors serendipitously isolated the ZMGRP promoter "from a maize B73 size-selected lambda genomic DNA (gDNA) library while attempting to isolate a second maize promoter, designated A3" (Spec., p. 111). Example 1 reports (Spec., p. 112):

> The analysis revealed that the restriction map and hybridization pattern of the putative clone was highly similar to, but not identical to, the expected A3 pattern. Partial sequencing of the clone revealed that the 5' sequence was highly homologous, but not identical to that of the A3 5' region. A GenBank search revealed that the 4000 base pair cloned sequence shared homology in

about the 400 most 3' based pairs with an ABA-inducible genomic clone reported by Gomez et al. (1988)(GenBank Accession Number X12564).

Example 2 characterizes the ZMGRP promoter constructs used in subsequent examples of transient expression analyses and transformations therewith. Example 2 in its entirety reads (Spec., pp. 112-113)(emphasis added):

> Sequence characterization of the ZMGRP promoter-containing plasmid revealed that the ~ 4.0 kb SacI insert contained a HindIII site 97 bp from the 5' end of the insert and approximately 360 bp of ZMGRP coding sequence 3' of the ATG start codon. Restriction enzyme analysis determined that there was a unique XhoI site approximately 400bp 5' of the ATG start site. The sequence around the XhoI site was determined and used to design a 5' PCR primer. A 3' PCR primer was designed to change the sequence around the ATG start site to create an NcoI site and to introduce a SmaI site 4 bp 5' of the ATG start codon. These primers were used to PCR amplify the DNA at the 3' end of the promoter from the XhoI site to the newly created NcoI site. The PCR fragment was used in a three way ligation, employing a HindIII to XhoI fragment containing the 5' ~3.2kbp part of the ZMGRP promoter region, the XhoI to NcoI fragment containing the 3' 0.4 kbp part of the ZMGRP promoter region, and the gus-nos sequence containing vector pGN73, which had been digested with HindIII and NcoI. The resulting construct was designated pZMGRP-GN73 (Fig. 2, SEQ ID NO:2)[5]. A construct designated pZMGRP-Act2-int-GN73 was made by replacing the SmaI - NcoI region of the ZMGRP promoter with a PvuII - NcoI restriction fragment from pDPG836 containing a rice Act2 intron 1 deletion derivative (Act2-int)(Fig. 1, SEQ ID NO:3)[6].

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[5] SEQ ID NO:2 includes 8076 nucleotides (Raw Sequence Listing (Paper No. 4)).

[6] SEQ ID NO:3 includes 9002 nucleotides (Raw Sequence Listing (Paper No. 4)).

Example 3 (Transient Expression Analysis of ZMGRP Promoter Function) describes the transient expression assays used for analysis of (Spec., p. 113):

> . . . expression of the gus reporter gene (E. coli beta-glucuronidase) fused to the ZMGRP promoter with an actin 2 intron (U.S. Serial No. 09/312,304) (ZMGRP (639) act 2 pGN73, FIG. 1) or without any intron (ZMGRP (639) pGN73, FIG. 2) . . . .

Having considered the teaching in appellants' specification, we now focus on the examiner's reasons to doubt the objective truth of the statements contained therein. In re Marzocchi, 439 F.2d 220, 223-24, 169 USPQ 367, 369-70 (CCPA 1971), instructs (footnote omitted):

> As a matter of Patent Office practice . . . a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. . . .
>
> . . . Most often, additional factors, such as the teachings in pertinent references, will be available to substantiate any doubts that the asserted scope of objective enablement is in fact commensurate with the scope of protection sought and to support any demands based thereon for proof. . . . [I]t is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or

> reasoning which is inconsistent with the contested statement.

To satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, for the full scope of contiguous nucleotide sequences appellants claim,

> . . . the specification [must] . . . enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation." <u>In re Wands</u>, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That <u>some</u> experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." <u>Id.</u> at736-37, 8 USPQ2d at 1404.

<u>In re Vaeck</u>, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991). As explained in <u>In re Wands</u>, 858 F.2d at 737, 8 USPQ2d at 1404 (footnotes omitted), "Enablement is not precluded by the necessity for some experimentation such as routine screening." <u>In re Wands</u>, 858 F.2d at 737, 8 USPQ2d at 1404, amplified the statement with a quote from <u>Ex parte Jackson</u>, 217 USPQ2d 804, 807 (Bd. App. 1982)(emphasis added):

> The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

<u>In re Wands</u>, 858 F.2d at 737, 8 USPQ2d at 1404, instructs (footnote omitted):

fragment of the whole 3536 bp nucleotide appellants describe, and the 639 bp fragment is defined solely in terms of its enzymatic restriction sites. The number of promoters which have been used to effect expression of phenotypic genetic codes appears to be small, yet appellants serendipitously have discovered one which preferably promotes expression of a gene to which it is fused only when a plant transformed by the construct is stressed and disclosed its sequence and functional properties in the application before us. The claims are narrowly limited to promoters having 3536 contiguous named nucleotides and functionally effective fragments thereof having at least 95 contiguous nucleotides. The prior art cited by the examiner relating to the activity and requisite functional sequences of the cauliflower mosaic virus 35S and nopaline synthase (nos) promoters does show that the nucleotide structure essential for promoter activity is highly unpredictable. However, the same prior art shows that the level of skill and knowledge in this art is extremely high, that mutations and deletions to the basic sequence more often than not lower rather than eliminate promoter activity, and that it is well within the ordinary skill of the artisan to determine those nucleotide sequences which are critical for functional activity.

Having considered all the evidence, we are not convinced that the examiner has satisfied her burden to show that, in light of the guidance and direction provided by appellants' specification, the kind and amount of experimentation required of one skilled in the art to make and use the full scope of the subject matter claimed is more than routine.

More significantly, however, the examiner urges (EA 8-9) (emphasis added):

> Appellants need to provide sufficient guidance for one skilled in the art to determine which of the claimed subfragments of SEQ ID NO:1 would be likely to have promoter function. In the absence of such guidance, it would require undue experimentation for one skill[ed] in the art to practice the claimed invention, because the ability of a particular nucleic acid sequence to function as a promoter is highly unpredictable on the basis of nucleotide sequence information alone. . . . The examiner maintains that <u>to provide sufficient guidance for one skilled in the art to determine which sequences have promoter function, the specification must provide some indication of what specific nucleotides the sequences must retain in order to retain promoter function. Appellants need not describe why or how the invention works in order to provide such guidance</u>.

Also see the examiner's rationale below (EA 11):

> The examiner does not assert that one skilled in the art would be without sufficient guidance in obtaining the claimed contiguous subfragments because the specification does not provide sufficient structural and functional information to prepare the recited sequences. The examiner asserts that one skilled in the art would be without sufficient guidance in recognizing the claimed contiguous subfragments that

examiner's answer, by In re Angstadt, 537 F.2d 498, 503-04, 190 USPQ 214, 218-219 (CCPA 1976):

> If . . . the disclosure must provide "guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction, whether the claimed product will be obtained" (emphasis in original), as the dissent claims, then all "experimentation" is "undue," since the term "experimentation" implies that the success of the particular activity is uncertain. Such a proposition is contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts. To require disclosures in patent applications to transcend the level of knowledge of those skilled in the art would stifle the disclosure of inventions in fields man understands imperfectly, like catalytic chemistry. The Supreme Court said it aptly in Minerals Separation, Ltd. v. Hyde, 242 U.S. 261, 270-271 (1916) . . . :
>
> > . . . the certainty which the law requires in patents is not greater than is reasonable, having regard to their subject matter . . . .
>
> Appellants have broadly disclosed a class of catalyst complexes whose use they deem to be part of the invention. But for this disclosure the public may have been deprived of the knowledge . . . . In this art, the performance of trial runs using different catalysts is "reasonable," even if the end result is uncertain . . . .

We have considered the examiner's explanations, appellants' responses, and all the evidence for and against the patentability of appellants' claims under 35 U.S.C. § 112, first paragraph, in light of the guidance our reviewing courts have provided.

Accordingly, we reverse the examiner's final rejections of Claims 1, 4-54, and 85-131 for noncompliance with description and enablement requirements of 35 U.S.C. § 112, first paragraph.

Conclusion

For the reasons stated herein above, it is

ORDERED that the examiner's rejections of Claims 1, 4-54, and 85-113 for noncompliance with the written description requirement of 35 U.S.C. § 112, first paragraph, is REVERSED; and

FURTHER ORDERED that the examiner's rejections of Claims 1, 4-54, and 85-131 for noncompliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, is REVERSED.

REVERSED

SHERMAN D. WINTERS Administrative Patent Judge	)	
TEDDY S. GRON Administrative Patent Judge	)	BOARD OF PATENT APPEALS AND INTERFERENCES
LORA M. GREEN Administrative Patent Judge	)	

Robert E. Hanson
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The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte NANCY HOUMARD, LUCILLE B. LACCETTI, ALBERT P. KAUSCH and EMIL M. OROZCO, JR. (hereafter Houmard)

Appeal No. 2005-0409
Application No. 09/757,089[1]

Before GRON, GRIMES and GREEN, Administrative Patent Judges.

GRON, Administrative Patent Judge.

DISCUSSION, REMAND and ORDER

We have before us an appeal under 35 U.S.C. § 134 of an examiner's final rejections of Claims 1, 5-12, 17-19, 22-29, 31-35, 38, 42-51, 82, 85-92, 94-98, 101, 105-123, 126 and 127 of Application 09/757,089 under 35 U.S.C. § 112, first paragraph, for noncompliance with both its written description and enablement requirements (Examiner's Answer, p. 2 (EA2)). Claims 1 and 5-13 presently pending in Application 09/757,089 are reproduced below:

---

1 Application for patent filed January 9, 2001.

1. An isolated nucleic acid comprising a maize chloroplastic F16BP aldolase promoter, said promoter comprising from 95 to 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

5. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 110 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

6. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 125 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

7. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 250 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

8. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 400 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

9. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 750 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

10. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1000 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

11. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1500 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

12. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises from about 1750 to about 1864 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:2.

13. The isolated nucleic acid of claim 1, wherein said chloroplastic F16BP aldolase promoter comprises the nucleic acid sequence of SEQ ID NO:2.

Appellants' Brief On Appeal was received in Tech Center 1600/2900 on July 9, 2003. The Examiner's Answer was mailed January 8, 2004.

On March 10, 2004, Appellants filed a Reply Brief accompanied by a copy of the August 29, 2003, decision of the Board of Patent Appeals and Interferences in Appeal No. 2003-0936 under 35 U.S.C. § 134 reversing an Examiner's Final Rejection of Claims 1, 4-54, and 85-131 of Application 09/532,806, filed March 21, 2000, under 35 U.S.C. § 112, first paragraph, also for noncompliance with its written description and enablement requirements. Application 09/532,806 of Appeal No. 2003-0936 and Application 09/757,089 of this appeal are commonly assigned to Monsanto Technology LLC. LUCILLE B. LACCETTI and EMIL M. OROZCO are named coinventors of the subject matter claimed in both applications, and each application was examined in Tech Center 1600. Claims 1 and 4-14 of Application 09/532,806 of prior Appeal No. 2003-0936 are reproduced below:

1. An isolated nucleic acid comprising a maize GRP promoter comprising at least 95 contiguous bases of SEQ ID NO:1.

4. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 110 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

5. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 125 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

6. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 250 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

7. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 400 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

8. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 750 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

9. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

10. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 1500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

11. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

12. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 2500 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

13. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises from about 3000 to about 3536 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:1.

14. The isolated nucleic acid of claim 1, wherein said GRP promoter comprises the nucleic acid sequence of SEQ ID NO:1.

Appellants informed the examiner in Part VII of the Reply Brief, <u>Summary of the Reply</u> (RB3-4):

> The same written description and enablement issues on appeal were recently decided by the Board in U.S. Patent Appl. Ser. No. 09/532,806. The specification and claims in that case were substantively the same as in this case. Both applications claimed a maize promoter sequence including fragments of at least 95 bp of the full length sequence and were rejected by the Examiner for an alleged lack of written description and enablement. The positions taken by the Examiner were essentially the same as in this case and were handled by the same Supervisory and Primary Examiners. The Board reversed the Examiner on both rejections in the '806 application, noting that the claims had literal support in the specification and that the substantial guidance provided by the specification rendered any experimentation needed to practice the full scope of the claimed invention routine. Given that the same substantive facts and legal issues are presented on appeal here, Appellants respectfully request that the same analysis be applied by the Board and the Examiner be reversed.
>
> The Answer fails to address the shortcomings of the written description rejection noted in Appellants' Brief. . . . .

The examiner responded to Appellants' Reply Brief by entering it, noting that the Reply Brief with the prior decision of the Board attached had been considered, and forwarding the application upon which the present appeal is based to the Board:

> The Reply Brief filed 3/10/2004 has been entered and considered. The application has been forwarded to the Board . . . for decision on the appeal.
>
> Any inquiry concerning this communication or earlier communications from the examiner should be directed to Stuart F. Baum . . . .

We understand that inquiries whether specifications satisfy the requirements of the first paragraph of 35 U.S.C. § 112 for the full scope of the claimed subject matter are claim and fact specific. Each case must stand on its own facts. Vas-Cath Inc. V. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Nevertheless, prior decisions of the Board provide invaluable instruction and guidance, especially where, as here, there are marked similarities between the presentations of the subject matter claimed, the disclosures in the supporting specifications, and the acknowledged skill and state of the art. Moreover, the cases allegedly have common assignees, common inventors and common examiners.

It is a hallmark of our legal system that similar cases are treated similarly. We do not, and no participant in this time-honored system may, casually disregard the outcome of cases with similar facts in prior decisions. Accordingly, we enter the following order.

Order

It is ORDERED that Application 09/757,089 is remanded to the primary examiner in charge of prosecution for reconsideration of the prior decision of the Board in Appeal No. 2003-0936, entered August 29, 2003, and comparison of the claimed subject matter, supporting disclosures, and acknowledged skill and state of the prior art in the applications relating to Appeal 2003-0936 and this appeal; and

It is FURTHER ORDERED that after reconsidering the prior decision of the Board in Appeal No. 2003-0936, entered August 29, 2003, and comparing the claimed subject matter, supporting disclosures, and acknowledged skill and state of the prior art in the applications relating to Appeal 2003-0936 and this appeal, should the examiner maintain the rejections presently appealed, the examiner shall not only reply to each and every point raised in appellants' Reply Brief citing and/or relying on the Board's prior decision in Appeal No. 2003-0936, but the examiner also shall distinguish the facts and law of the two appeals which support a decision in this appeal contrary to our decision in Appeal No. 2003-0936.

This application, by virtue of its "special" status, requires an immediate action. Manual of Patent Examining Procedure § 708.01 (8th ed., rev. 2, May 2004). It is important that the Board be informed promptly of any action affecting the appeal in this case.

REMAND

TEDDY S. GRON Administrative Patent Judge	)	
ERIC BURTON GRIMES Administrative Patent Judge	)	BOARD OF PATENT APPEALS AND INTERFERENCES
LORA M. GREEN Administrative Patent Judge	)	

TSG/jlb